UNIVERSITÄT
DUISBURG
ESSEN

EARLY MOLECULAR CHANGES IN ARSENIC EXPOSED HUMAN UROTHELIAL CELLS DEPENDING ON CELLULAR UPTAKE AND BIOTRANSFORMATION

Inaugural Dissertation

for the degree of

Doctor of Natural Science

– Dr. rer. nat. –

Presented by

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A Thesis presented to

The Faculty of Chemistry

University of Duisburg-Essen

Germany

2013

Die vorliegende Arbeit wurde im Zeitraum von August 2008 bis August 2013 im Arbeitskreis von Prof. Dr. Elke Dopp am Institut für Hygiene und Arbeitsmedizin der Universität Duisburg-Essen durchgeführt.

Tag der Disputation: 30. Januar 2014

Gutachter:	Prof. Dr. Elke Dopp
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Vorsitzender:	Prof. Dr. Barcikowski

List of Publications

The present work supported by a grant of the DFG (Deutsche Forschungsgemeinschaft) to ED (DO 332/8-1), AH (HI 276/16-1) and GJ (JO 753/2-1) and parts of it were already published:

Research article

Johnen G, Rozynek P, van der Gathen Y, Bryk A, Zdrenka R, Johannes C, Weber DG, Igwilo-Okuefuna O, Raiko I, Hippler J, Brüning T, Dopp E (2013) Cross-contamination of a UROtsa stock with T24 cells – molecular comparison of different cell lines and stocks. *Plos one*, Vol. 8, No. 5, doi: 10.1371/journal.pone.0064139

Hippler J, Zdrenka R, Reichel RAD., Weber DG, Rozynek P, Johnen G, Dopp E & Hirner AV (2011) Intracellular, time-resolved speciation and quantification of arsenic compounds in human urothelial and hepatoma cells. *Journal of Analytical Atomic Spectrometry*, Vol. 26, No. 12, pp. 2396-2403

Book chapter

Zdrenka R, Hippler J, Johnen G, Hirner AV & Dopp E (2012) Intracellular Arsenic Speciation and Quantification in Human Urothelial and Hepatic Cells. In: *Bladder Cancer - From Basic Science to Robotic Surgery*, Canda AE (Ed.), ISBN: 978-953-307-839-7, InTech

Zdrenka R, Hippler J, Weber DG, Braescher M Johen G, Hirner AV & Dopp E (2010) Early molecular changes in the genome of arsenic-exposed human urothelial cells depending on cellular uptake and biotransformation. In: *Arsenic in Geosphere and Human Diseases*, Jean JS, Bundschuh J & Bhattacharya P (Eds.), ISBN: 978-0-415-57898-1, CRC Press

Hipppler J, Zdrenka R, Müller SD, Reichel RAD, Braescher M, Dopp E & Hirner AV (2010) Intracellular speciation of arsenic compounds in human urothelial and hepatic cells. In: *Arsenic in Geosphere and Human Diseases*, Jean JS, Bundschuh J & Bhattacharya P (Eds.), ISBN: 978-0-415-57898-1, CRC Press

Published Abstracts

Johnen G, Weber DG, Zdrenka R, Schreiber J, Rettenmeier AW, Bruening T & Dopp E (2009) Verminderte COX2-Expression in Arsen-exponierten UROtsa-Zellen korreliert mit einer Erhöhung von regulierenden microRNAs. *Arbeitsmed. Sozialmed. Umweltmed.,* Vol 44, No. 3, pp. 196-197

The results were further presented as talks on the following scientific conferences:

Zdrenka R, Hippler J, Weber D G, Rozynek P, Johnen G, Hirner A V & Dopp E (2011) Intracellular, time resolved arsenic speciation in human urothelial and hepatoma cells. *Metallomics 2011*, Muenster, Germany, June 15-18 2011

Zdrenka R, Hippler J, Johnen G, Hirner AV & Dopp E (2011) Biotransformation and genotoxic effects of monomethylarsonous acid [MMA(III)] in methy-lating and non-methylating human cells. *GMS*, Leipzig, Germany, October 7-9 2010

Zdrenka R, Hippler J, Weber DG, Braescher M Johen G, Hirner AV & Dopp E (2010) Early molecular changes in the genome of arsenic-exposed human urothelial cells depending on cellular uptake and biotransformation. *AS2010 The third International Congress on Arsenic in the Environment,* Tainan, Taiwan, May 17-21 2010

Moreover, data from the present work were presented as posters on the following scientific conferences:

Zdrenka R, Hippler J, Johnen G, Hirner AV & Dopp E (2011) Biotransformation and genotoxic effects of monomethylarsonous acid [MMA(III)] in methy-lating and non-methylating human cells. *EnTox*, Dortmund, Germany, May 19-20, 2011 (**Best Poster Award**)

Zdrenka R, Hippler J, Johnen G, Hirner AV & Dopp E (2011) Arsenic-induced malignant transformation of human urothelial cells. *xCELLigence System Global User Conference,* Rome, Italy, May 3-4 2011

Zdrenka R, Hippler J, Johnen G, Hirner AV & Dopp E (2011) Biotransformation and genotoxic effects of monomethylarsonous acid [MMA(III)] in methy-lating and non-methylating human cells. *GMS*, Leipzig, Germany, October 7-9 2010 (**Best Poster award**)

Zdrenka R, Hippler J, Braescher M, Hirner AV, Rettenmeier AW & Dopp E (2009) Biotransformation and genotoxic effects of monomethylarsonous acid [MMA(III)] in methy-lating and non-methylating human cells, *ICEM-10th International Conference on Environmental Mutagens,* Florence, Italy, August 20-25 2009

Johnen G, Weber DG, Zdrenka R, Schreiber J, Rettenmeier AW, Bruening T & Dopp E (2009) Verminderte COX2-Expression in Arsen-exponierten UROtsa-Zellen korreliert mit einer Erhöhung von regulierenden microRNAs. *Deutsche Gesellschaft fuer Arbeitsmedizin und Umweltmedizin e.V. 49. Jahrestagung*, Aachen, Germany, March 11-14 2009

Reichel RAD, Braescher M, Zdrenka R, Hippler J, Dopp E & Hirner AV (2009) Speciation of arsenic compounds after uptake into human urothelial cells. *11th JCF-Fruehjahrssymposium*, Essen, Germany, March 11-14 2009

Terms and Abbreviations

AP-1	Activator protein 1
Apaf-1	Apoptotic protease activating factor 1
APS	Adenosine 5' phosphosulfate
As(III)	Arsenite
As(V)	Arsenate
ATL buffer	Tissue Lysis Buffer
ATP	Adenosine triphosphate
BCA	Bicinchoninic acid
BCL2	B-cell lymphoma 2
BPE	Bovine pituitary extract
BSA	Bovine serum albumine
CAM Assay	Chicken Chorioallantoic-Membrane Assay
CDK	Cyclin-dependent kinase
cDNA	complementary DNA
CI	Cell Index
COX-2	Cyclooxygenase-2
CpG site	regions of DNA where a cytosine nucleotide occurs next to a guanine nucleotide
DMA(III)	Dimethylarsinic acid
DMA(V)	Cacodylic acid
DMPS	2,3-Bis(sulfanyl)propane-1-sulfonic acid
DNA	Deoxyribonucleic acid
DNMTs	DNA methyltransferases
dNTP	Desoxyribonucleotide triphosphate

EDTA	2-[2-[Bis(carboxymethyl]amino]ethyl-(carbomethyl)amino]acetic acid
EGFR	Epidermal-growth-factor-receptor
ELISA	Enzyme-linked immunosorbent assay
G1-phase	First gap phase (in cell cycle)
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
gDNA	Genomic DNA
H_2O_2	Hydrogen peroxide
HepG2	Hepatocellular carcinoma cell line
HPLC	High performance liquid chromatography
HRP	Horseradish peroxidase
HUEPC	Primary human urothelial epithelial cells
ICP/MS	Inductive coupled plasma mass spectrometry
IARC	International Agency for Research on Cancer
LINE	Long interspersed nuclear element
m/z	mass-to-charge ratio
MAPK	Mitogen activated protein kinase
MeCP2	Methyl-CpG-binding protein 2
MEM	Earle's minimal essential medium
MGMT	O^6-methylguanine-DNA methyltransferase
miR	MicroRNA
MMA(III)	Monomethylarsonous acid
MMA(V)	Monomethylarsonic acid
PARP	Poly (ADP-ribose) polymerase
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
RAR ß	Retinoic acid receptor ß
RASSF1	Ras Association Domain family 1
RB	Retinoblastoma protein

RISC	RNA-induced silencing complex
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RPE	mild washing buffer for miRNA isolation
S-phase	Synthesis phase (in cell cycle)
SAEC	Small airway epithelial cells
SAM	S-adenosyl methionine
SEM	Standard error of mean
T3	3,5,3'-Triiodothyronine
TMAO	Trimethylarsine oxide
TMB	3,3′,5,5′-Tetramethylbenzidine
UROtsa	SV40 immortalised human urothelial cell line
WHO	World Health Organization
8-Oxo-dG	8-Oxo-2'-deoxyguanosine
AS3MT	Arsenic (+3 oxidation state) methyl transferase

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1 Summary

Millions of people are highly exposed to arsenic, especially via contaminated drinking water. Once entered the human body, arsenic underlies (hepatic) metabolism leading to various methylated species, until it is either accumulated in skin and other tissues, or finally excreted via the renal pathway. Common health consequences are, among others, hyperkeratosis, vascular diseases, and cancer. Especially environmentally relevant doses of arsenic exhibit a highly carcinogenic potential. Arsenic-induced lung cancer, as well as skin, kidney, and bladder cancer have been reported. Bladder cancer in general was reported to occur very frequently with a high recurrence rate; it is in fifth place of the most commonly diagnosed cancer diseases and on second place of those observed in the urogenital tract (Zimbardi et al., 2012). Thus, this study was conducted to focus on the mechanisms of arsenic-induced bladder cancer. Since carcinogenesis is a very complex process with highly species- and tissue-specific mechanisms, an *in vitro* test system was selected to assay molecular mechanisms during arsenic-induced bladder carcinognesis, taking advantage of an isolated cellular system instead of the complexicity of a whole living organism.

The aim of the study was to give an overview over some of the most relevant key events during arsenic-induced carcinogenesis, occurring as a consequence of short-term and chronic low-dose exposure by consolidating interdisciplinary research. To better understand the underlying mechanisms of arsenic carcinogenicity, studies were carried out to correlate arsenic metabolism and genotypic effects with epigenetic modifications and phenotypical alterations under chronic exposure conditions up to 90 weeks. Hence, it was important to detect and analyse intracellular arsenic species and their metabolic products. Therefore, the cellular uptake of arsenic species in non-methylating human urothelial cells (T24) in comparison to methylating human hepatic cells (HepG2) was investigated, and the intracellularly detected arsenic was speciated and quantified. Arsenic-induced genotoxic effects in T24 cells were measured by means of the Alkaline Comet Assay, and the malignant transformation after chronic arsenic treatment up to 90 weeks was assayed using the *in vitro* Mammalian Cell Transformation Assay, the Colony Formation Assay, and the Migration and Invasion Assay. MicroRNA analysis and examination of altered DNA methylation patterns were carried out to determine the arsenic-induced modulation of

epigenetic regulation systems. Moreover, COX-2 protein, which is frequently reported to be increased in malignant tissues and therefore considerd to be an important molecula marker, was analysed. Since the various arsenic species emerging from the hepatic biotransformation of arsenic exhibit distinctly different toxicity, this study focussed especially on effects induced by the methylated metabolite monomethylarsonous acid (MMA(III)), which was recently shown to be one of the most cyto-and genotoxic metabolites. The present study aims to enhance the understanding of arsenic-induced toxicity and carcinogenesis in the urinary bladder epithelium.

The experiments conducted within the present study revealed a rapid cellular uptake of MMA(III) in both T24 and HepG2 cells, followed by subsequent conjugation to proteins and other cellular structures. While in HepG2 cells MMA(III) was further methylated to dimethylated arsenic due to the metabolic capacity of these cells, no such biotransformation was observed in T24 cells. Nevertheless, in both cellular systems oxidation to pentavalent species was observed, leading to the assumption that autophagy of affected cellular structures by utilising oxidative processes occurred. This was further confirmed by the presence of unconjugated trivalent arsenic species, but to a minor degree.

Genotoxicity of the trivalent arsenic species, as well as arsenate was detected already after 30 min of exposure. Longer exposure durations revealed cytotoxicity, overlapping genotoxic effects. Genotoxicity was observed to be not only dependent on the oxidation state, but also on the methylation state, as genotoxicity of trivalent arsenic species increased with the number of methyl groups. As a consequence and due to their biomethylation capacity, HepG2 cells appeared to be more sensitive towards the genotoxic potential of arsenic species when compared to T24 cells. Additionally, the study revealed that primary cells such as human urothelial epithelial cells (HUEPC) are more susceptible to arsenic-induced genotoxicity than permanent cell lines.

Furthermore, to investigate whether MMA(III) is only a genotoxic carcinogen or if it also exhibits non-genotoxic molecular changes, the epigenetic effects on T24 cells after chronic low-dose treatment were investigated. Key events such as modification of DNA methylation and miRNA profiles, as well as COX-2 protein activation were analysed. The experiment revealed that in T24 cells, under the chosen test

conditions, exposure to MMA(III) led to the alteration of miR-15a, -19a, 19b, 30a-3p, -126, -128a, -139-5p, -146a, -200a, and -429; which are known to correlate with the occurrence of cancer diseases (Gregory et al., 2008; Hurst et al., 2009; Ichimi et al., 2009; Lin te al., 2009). This indicates that altered miRNA profiles might play a pivotal role in MMA(III)-induced further malignant transformation of T24 cells, which was phenotypically verified in experiments described below. In contrast, altered DNA methylation was only observed for C1QTNF6 and CDH1. Nevertheless, since T24 cells are transitional cell carcinoma cells of the urinary bladder, they show a distinctly altered DNA methylation background. Hence, it cannot conclusively be excluded that those changes overlay the effects caused by MMA(III) exposure.

Elevated COX-2 protein levels were observed throughout the chronic low-dose exposure to MMA(III). It can be assumed from the results that the regulation of the COX-2 protein is not accomplished by the amount of mRNA, but presumably by the interaction of COX-2 mRNA with miR-26a and -26b. Further experiments are necessary to confirm this and to unveil the respective mechanism.

Among the molecular effects of chronic low-dose exposure to MMA(III) also the development of altered phenotypic appearance was investigated. The determination of the *in vitro* Mammalian Cell Transformation Test, the Colony Formation Assay, and the Migration and Invasion Assay revealed further malignant transformatioin of T24 cells after long-term exposure to MMA(III). In addition to morphological changes, the loss of contact inhibition and anchorage dependent growth were observed. Moreover, T24 cells chronically treated with MMA(III) developed the ability of migration and invasion, which can be concluded to be the basis for metastasizing properties *in vivo*.

In summary, MMA(III) was shown to be rapidly taken up and to exhibit both genotoxicity, as well as tumour progression (i.e. non-genotoxic carcinogenic activities such as COX-2 protein activation, altered miRNA expression profiles, and altered DNA methylation), which resulted in a further malignant transformation of T24 carcinoma cells after chronic low-dose exposure up to 90 weeks. This study indicated various molecular modes of action of MMA(III) *in vitro*, which only could be analysed and correlated to each other by consolidating interdisciplinary research. Nevertheless, the results must be further confirmed *in vivo* to be conclusively correlated to the MMA(III)-induced development of bladder cancer.

2 Introduction

2.1 The chemistry of arsenic

Arsenic occasionally occurs elemental as “Scherbenkobalt”, but it can generally be found in anionic or cationic bindings. Arsenic presents its non-metallic character in the anionic compounds (metal-arsenides, e.g. “Arsenkies” $FeAs_2 \cdot As_2S_3$) and its metallic character in the cationic compounds (arsenic sulphides, e.g. “Auripigment” As_2S_3, or arsenic oxides, e.g. “Arsenolithe” As_2O_3). Arsenic can be isolated by heating “Arsenkies” in the absence of air, whereas arsenic is sublimated (Equation 1) (Hollemann & Wiberg, 1964).

(1) $$FeAsS \longrightarrow FeS + As$$

The elemental arsenic occurs in three different modifications. The grey arsenic consists of undulated hexagons forming tight, interacting bilayers. The interaction between the atoms of the different layers results in an electroconductive property. This high-polymeric substance melts and vaporizes only under the loss of its structure (Hollemann & Wiberg, 1964). By heating under standard pressure at 633°C the grey arsenic sublimates without melting in the form of As_4 molecules, at > 800°C the steam consists of As_2. Yellow arsenic is prepared by rapidly cooling down the sublimated grey arsenic. It is metastable and consists of tetraedric As_4 molecules. A fast transition into the grey arsenic can be observed. By condensation of gaseous arsenic on 100°C – 200°C warm surfaces black arsenic can be prepared. It exists in different amorphous forms, which are hard and brittle. Although black arsenic forms bilayers, it is non-conductive because the layers are of an irregular structure and of a lesser density than in grey arsenic, so there are no interactions. Arsine (AsH_3) can be formed by nascent hydrogen reacting with soluble arsenic compounds (Equation 2) (Hollemann & Wiberg, 1964), e.g.

(2) $$As(OH)_3 + 6\,H \rightarrow AsH_3 + 3\,H_2O.$$

This reaction became popular as the wet chemical analysis for arsenic and is known as the Marsh test. The most important halids are of the type AsX_3 and AsX_5 (X = halogen), which can easily be obtained out of the elements (Hollemann & Wiberg, 1964).

Organic arsenic compounds usually occur in plants and animals. Cacodyl was the first synthetically produced arsenic species, when Louis-Claude Cadet de Gassicourt 1757 tried to synthesise invisible ink for the military. Hereby, Cacodyl was formed as a smoky and smelly fluid (Elschenbroich, 2008), but the structure was not explored until 1858, when Robert Bunsen described it as tetramethyldiarsenic ($(CH_3)_2As$-$As(CH_3)_2$) (Seyferth, 2001).

Trimethylarsine (Gosio-Gas) is the basic organic arsenic compound. Today there are many organo-arsenic species known (Garje & Jain, 1999). The bond between carbon and arsenic is generally stable against water and alkylated arsenic acids ($RAsO(OH)_2$ such as monomethylarsonic acid and $R_2AsO(OH)$ like dimethylarsinic acid or Cacodylic acid) can easily be prepared by reaction of sodium arsenite and alkyl halids (Meyer reaction). Analogue aryle arsenicals are generally synthesised using the Bart Reaction with sodium arsenite and diazonium salts (Goates et al., 1972). The trivalent arsenic species can be built by reduction of the homologous pentavalent compound using sulphur dioxide (SO_2) (Styblo et al., 1997a). Arsenosugar (Arsinoylribosides) can be found in algae, whereas in fish and seafood predominantly Arsenobetaine and Arsenocholine as well as other methylated species can be detected (Edmonds & Francesconi, 2003; Kuehnelt & Goessler, 2003).

2.2 Arsenic application and human exposure

In the ancient world arsenic was the most famous murderous poison with a lethal dose of 100 – 200 mg. In the 19th century so called “Arsenic Eaters” consumed arsenic to keep well and fit and to increase their potency (Schäfer, 1861; Cullen, 2008). Furthermore, arsenic was part of green pigments in tapestry (Schweinfurt green), but people were already warned of the use at that time (Chasteen et al., 2002).

100 years ago arsenic became famous as Salvarsan, which was developed by Paul Ehrlich as medicine against syphilis. It saved many lives until it was finally replaced

by penicillin. Additionally, sodium hydrogenarsanilate, known as Atoxyl, was applied in the therapy of sleeping sickness, and until today arsenic is used in Chinese medicine (Cullen, 2008). Today, in chemotherapy arsenic is of particular importance. For example, it is known as TRISENOX (arsenic trioxide) for the cure of leukaemia (Antmann, 2001), which was authorised in 2002 (EPAR, 2007).

Arsenic is not only applied in medicine; elementary arsenic is used in alloys such as gallium arsenide GaAs) (Housecroft & Sharpe, 2006). Furthermore, it is part of wood preservatives in the form of chromated copper arsenate (Bringezu, 1990). In the USA the private application of arsenic containing wood preservatives is forbidden (U.S. EPA, 2002), and in the European Union the use is strongly regulated and limited (Amtsblatt der Europäischen Gemeinschaften, 2003).

In the agriculture arsenic can be found in pesticides and herbicides (sodium methylarsenate, dimethylarsinic acid), and is applied as the product Roxarson in the porcine and poultry mast to stimulate the growth of the animals (Chapman & Johnson, 2002).

The major source of exposure for human beings is arsenic contaminated groundwater. At many places on earth the drinking water contains concentrations above 10 µg/l, which significantly exceed the tolerable value recommended by the WHO (World health organization [WHO], 2001, as cited in Zdrenka et al., 2012[1]). This is considered as a health threat for millions of people, especially in Bangladesh, Vietnam, and Latin America (Ng et al., 2003, as cited in Zdrenka et al., 2012), where the considerable arsenic occurrence is of geogenic origin (erosion), related to mining activities, and geothermal waters (Fig. 1) (Smedly & Kinniburgh, 2002, as cited in Zdrenka et al., 2012).

[1] Available information on human exposure to arsenic was recently reviewed by Zdrenka et al. (2012). The respective information was interspersed into this chapter.

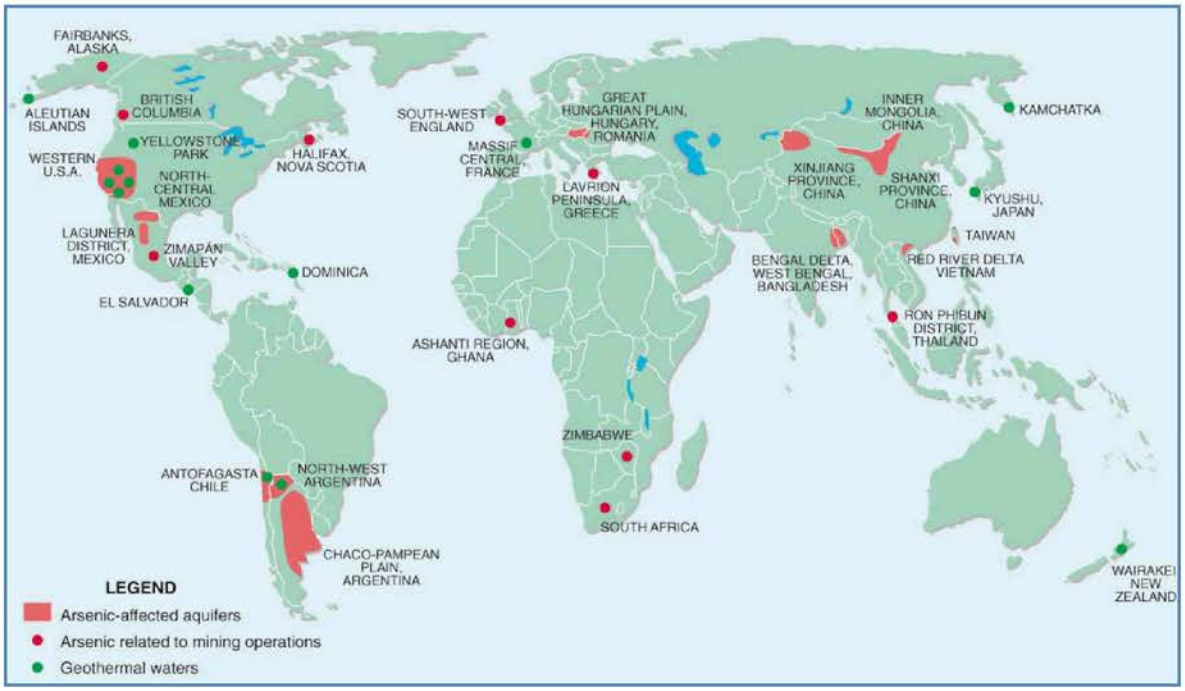


Fig. 1 Map of arsenic affected aquifers (Smedly & Kinniburgh, 2002)

The main arsenic species detected in drinking water are arsenite and arsenate. But the geothermal waters (Hot Spots) in the Yellostone Nationalpark, USA, predominantly contain several mg/l of methylated thioarsenicals such as mono-, di-, tri-, and tetrathioarsenate, as well as methylated arsenoxy-, and -thioanions (Planer-Friedrich et al., 2007, as cited in Zdrenka et al., 2012). In the surrounding atmosphere 0.5 – 200 mg/m^3 of volatile arsenic species can be detected, which have been identified, among others, as $(CH_3)_2AsCl$, $(CH_3)_3As$, $(CH_3)_2AsSCH_3$, and CH_3AsCl_2 (Planer-Friedrich et al., 2006, as cited in Zdrenka et al., 2012).

A second important source of arsenic is the air, where only one third of the occurring arsenic is of natural origin. Further anthropogenic sources are ore mining, smelteries, and the combustion of fossil fuels (Lozna & Biernat, 2008, as cited in Zdrenka et al., 2012).

Among polluted air and contaminated drinking water also the human diet is of importance. For example, high doses of arsenic can be detected in fish, seafood, and algae, so that in 2004 the Food Agency of the UK warned against the consumption of Hijiki (hijikia fusiforme, black sea weed) (Food Standards Agency of the UK, 2004, as cited in Zdrenka et al., 2012) as it contains inorganic arsenic up to 100 mg/kg. High arsenic concentrations can be found in the urine of the consumers (Nakjima et al.,

2006, as cited in Zdrenka et al., 2012). Francesconi (2010, as cited in Zdrenka et al., 2012) published the detection of up to 50 different arsenic compounds in fish and seafood, whereas their toxicity still is widely unknown. Especially rice and rice products exhibit considerable noxious effects, as they contain high doses of toxic inorganic arsenic (Meharg et al., 2008; Signes-Pastor et al., 2009; Sun et al., 2009, as cited in Zdrenka et al., 2012) and form the nutrition base especially of the Asian people. But not only rice from Asia, but also rice from the middle of the USA is contaminated with arsenic, the latter sustaining its contaminant not basically from natural sources but from pesticides anciently used on the cotton plantations, until DDT (dichlorodiphenyltrichloroethane) was introduced to replace arsenic in biocides (Hirner & Hippler, 2011, as cited in Zdrenka et al., 2012). The following figure Fig. 2 gives an overview of the main pathways of human exposure to arsenic:

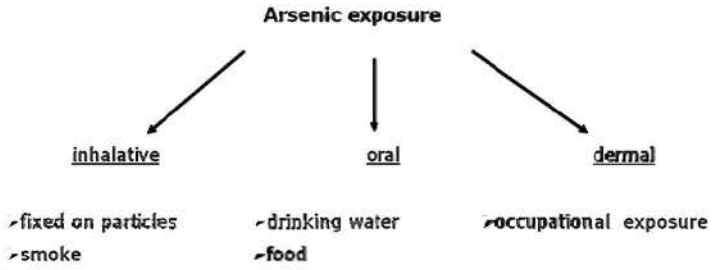


Fig. 2 Pathways of human exposure to arsenic (Dopp, 2007)

2.3 Biodisposition and biomethylation of arsenic

Chronic low-dose exposure may cause various diseases including bladder and other cancers. After ingestion arsenic is distributed to several organs, where it undergoes biotransformation (Zdrenka et al., 2012). Gosio was the first to observe the microbial conversion of inorganic arsenic to methylated species in 1891 (Gosio, 1892). He described the production of a gas with typical garlic odour. Later, Challenger and co-workers identified this gas as trimethylarsine (Challenger et al., 1933). In 1945 Challenger further postulated the first mechanism of arsenic biomethylation in yeast, and described it as based on repetitive reduction and oxidative methylation as shown in Fig. 2 (Challenger, 1945).

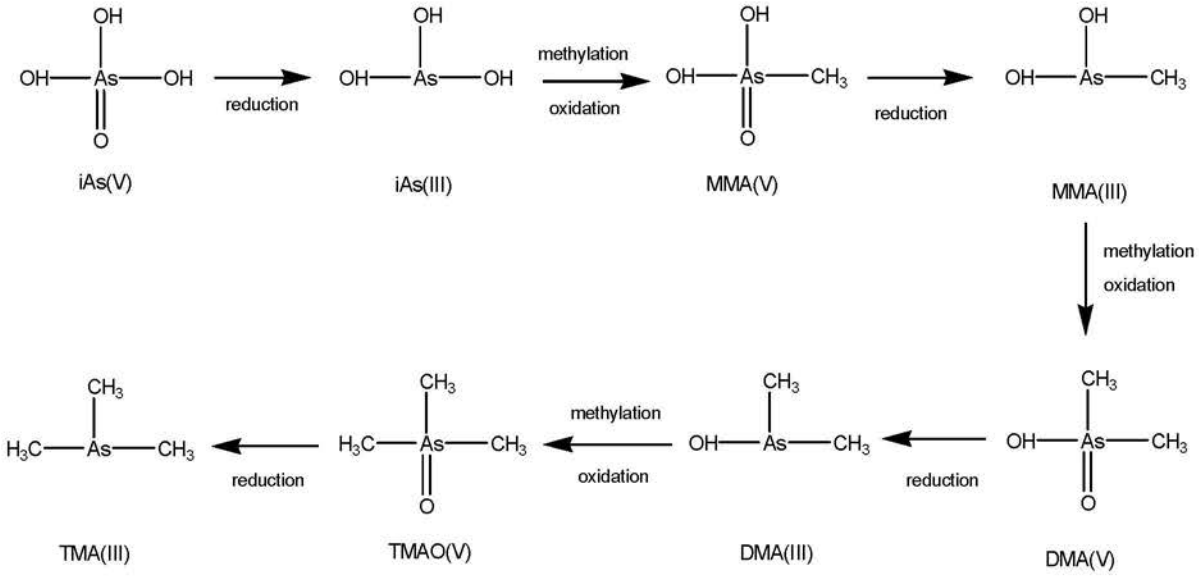


Fig. 3 Mechanism of arsenic biomethylation according to Challenger (Challenger, 1945)

In 1997 Aposhian published that this biomethylation can also be performed by bacteria and animal tissue (Aposhian, 1997), and in 2005 Hayakawa et al. suggested a metabolic pathway for arsenic in rat liver tissue homogenate as shown in Fig. 4; hereby, arsenic-glutathione complexes are formed, which are then methylated by arsenic methyltransferase (Cyt19) and S-adenosyl-L-methionine (SAM) (Hayakawa et al., 2005, as cited in Zdrenka et al., 2012[2]).

This biotransformation of arsenic is generally regarded as a detoxifying process, since the toxicity of monomethylarsonic acid (MMA(V)) and dimethylarsinic acid (DMA(V)) is much lower than that of inorganic arsenic (Hirano et al., 2003; Hirano et al., 2004). Nevertheless, the trivalent arsenic intermediates and metabolites such as monomethylarsonous acid (MMA(III)) and dimethylarsinous acid (DMA(III)) are considered to be the most cyto- and genotoxic species (Dopp et al., 2010a, as cited in Zdrenka et al., 2012).

[2] Available information on arsenic biodisposition and biomethylation was recently reviewd by Zdrenka et al. (2012). The respective information was interspersed into this chapter.

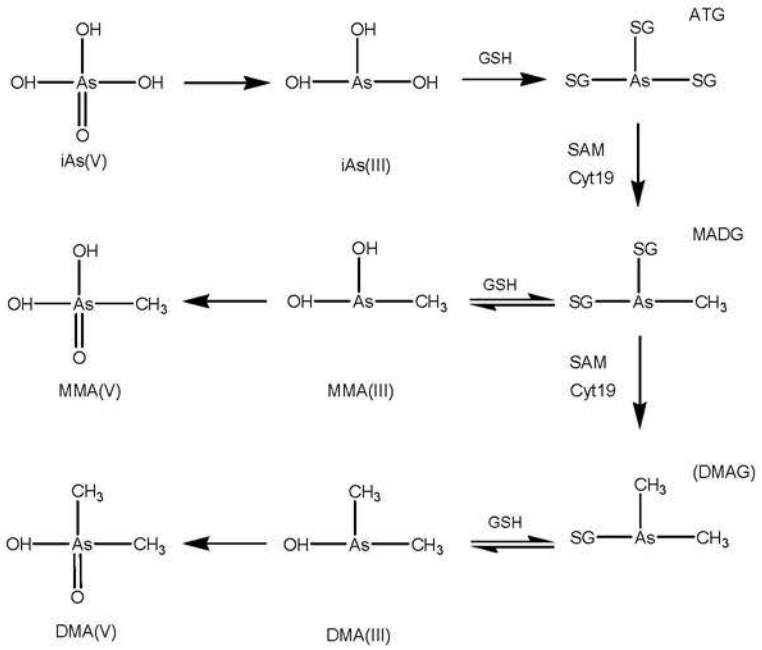


Fig. 4 Mechanism of arsenic biomethylation according to Hayakawa (Hayakawa et al., 2005, as cited in Zdrenka et al., 2012) GSH = reduced gluthatione, ATG = arsenic trigluthatione, MADG = monomethylarsonic digluthatione, DMAG = dimethylarsinic gluthatione, SAM = S-adenosyl-L-methionine, Cyt19 = arsenic methyltransferase

The liver is the main site of arsenic metabolism, and the renal route is the most important excretion pathway. A number of human studies have revealed that predominantly MMA(V) and DMA(V) can be detected in urine samples of arsenic-exposed individuals. Fillol et al. (2010, as cited in Zdrenka et al., 2012) reported that the main metabolites excreted in the urine were DMA(V) (84%) and MMA(V) (12%) and observed a significant correlation between the consumption of seafood and the excretion of arsenic metabolites in the urine. Furthermore, Aposhian et al. (2000, as cited in Zdrenka et al., 2012) detected concentrations of about 50 nM MMA(III) in the urine of an arsenic-exposed human population in Romania. In animals given the food additive Roxarson, arsenate was identified as the main metabolite present in the excrement; however the main part of the parent compound was found to be excreted as such, i.e., without any further conversion (Gabriano et al., 2003). Feldmann et al. (2000) analysed the urine of the seaweed-eating sheep living on an island in the north of Scotland. The author and his coworkers detected the metabolites of the ingested arsenosugar in the urine of these sheep and later also in the urine of human individuals. They showed, that arsenosugar is comparable to inorganic arsenic and accumulates in the different organs. Recently, the same group also demonstrated

that arsenobetaine is not as inert in the body, as it was expected to be so far (Newcombe et al., 2010). They corrected the misbelief that arsenobetaine is excreted without any metabolism and accumulation, and they showed that it can also be taken up by the body. Furthermore, they showed that arsenobetaine can also be formed out of DMA(V) and inorganic arsenic.

In the rat, studies have shown that ingestion of DMA(V) causes bladder cancer after chronic exposure (Wei et al., 1999, as cited in Zdrenka et al., 2012). Subsequent experiments revealed that a large number of secondary arsenic metabolites are formed and excreted via urine; for example, thiolated arsenics such as dimethylmonothioarsonic acid (DMMTA(V)) could be detected in the urine of DMA(V)-exposed rats (Yoshida et al, 1998, as cited in Zdrenka et al., 2012).

The liver is the main site of arsenic metabolism, and the renal route is the most important excretion pathway. Although most of the ingested arsenic is excreted again, residues accumulate in tissue and skin as shown in Fig. 5 (Dopp, 2007). The impact of the arsenic circulation in the blood stream was specified by Naranmandura et al. (2006, as cited in Zdrenka et al., 2012); these authors published that the arsenic metabolites DMA(III) (dimethylarsinous acid) and (dimethylmonothioarsinic) DMMTA(V) can be taken up by red blood cells and transformed into DMDTA(V) (dimethyldithioarsinic). Furthermore, they found that DMA(III) is bound to haemoglobin in rat red blood cells, but not in human red blood cells, indicating species specific differences of arsenic toxicity.

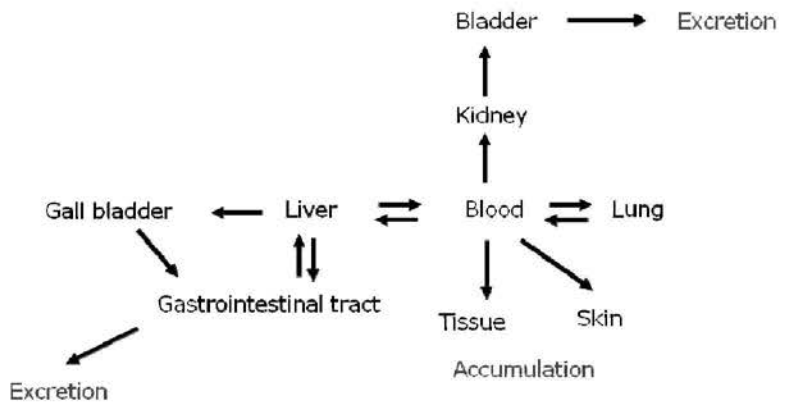


Fig. 5 Biodisposition of arsenic (Dopp, 2007)

It is still unknown, however, which of the various arsenic metabolites is responsible for the development of bladder cancer. The metabolism of arsenic is of great importance for toxicological studies. As summarized by Dopp et al. (2010b) and Hirner & Rettenmeier (2010), the cyto- and genotoxic effects are highly dependent on the particular arsenic species, its cellular uptake, and its intracellular metabolism (Zdrenka et al., 2012). For example, the toxicity of MMA(III) is 20 times higher than that of As(III) (Styblo et al., 2002; Bredfeldt et al., 2006, as cited in Zdrenka et al., 2012).

2.4 Toxicity of Arsenic

The availbale data on arsenic toxicity was recently reviewed and summarised by Zdrenka et al (2012). The following chapter recites this information.

Acute arsenic toxicity causes abdominal pain, nausea and faintness, vomiting, diarrhoea, and seizures (Gorby & Albuquerque, 1988, as cited in Zdrenka et al., 2012). In contrast, chronic arsenic toxicity is less conspicuous. Characteristic symptoms are Mees' lines and hyperkeratosis predominantly at palms and soles of the feet. Furthermore, vascular diseases and peripheral neuropathy, as well as, diabetes mellitus may occur (Smith et al., 2000, as cited in Zdrenka et al., 2012). Until now, more than 60 Mio people in Bangladesh and India are still at risk of arsenic-induced diseases due to arsenic concentrations of 10 - 50 µg/l or even higher (Chakraborti et al., 2004, as cited in Zdrenka et al., 2012). The mechanisms of arsenic toxicity are only poorly understood. The structural likeness of arsenate and phosphate (Fig. 6), which is called molecular mimicry, results for example in the use of the same cellular transporters. Furthermore, there are plenty of biomolecules known, in which arsenate replaces phosphate, such as arsenosugars, arsenolipids, and arsenobetaine (Rosen et al., 2011, as cited in Zdrenka et al., 2012).

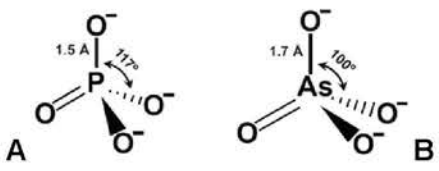


Fig. 6 Molecular mimicry of phosphate (A) and arsenate (B) (Rosen et al., 2011)

The relevance of this molecular mimicry is pointed out in the study of Wolfe-Simon et al. (2011, as cited in Zdrenka et al., 2012), reporting that phosphate can be substituted by arsenate in macromolecules of a bacterium, strain GFAJ-1 of the Halomonadaceae, isolated from Mono Lake, CA. However, these data were controversially discussed in the literature lately. Hereby, some points have to be considered: first the rapid hydrolysis of arsenate esters, and second the notably altered three dimensional structure of macromolecules like DNA (Rosen et al., 2011, as cited in Zdrenka et al., 2012).

In addition to acute effects of arsenic toxicity, chronic arsenic exposure is also associated with an increased risk of cancer, especially at environmentally relevant doses. In contrast to the temporary administration of high doses of arsenic in cancer therapy, chronic exposure to only small amounts can cause cancer. Especially arsenic-induced lung cancer, as well as skin, kidney, and bladder cancer were reported (Chiou et al, 1995; Chen et al., 2010; Tseng, 2007, as cited in Zdrenka et al., 2012). Hence, the following chapters will focus on the mechanisms of carcinogenesis in general, and the effects of chronic low dose exposure to arsenic and its influence of carcinogenesis.

2.5 Cancer and carcinogenesis

According to the WHO, cancer diseases are with 7.6 Mio fatalities (13%) part of the most common causes of death worldwide (WHO, 2006). Cancer diseases are multifaceted and can involve every organ or tissue. The frequency is, among others, dependent on age, gender, lifestyle, and diet (Fig. 7). The basis of cancer is the accumulation of aberrant and growth disinhibited cells depending on evolution and selection (Cahill et al., 1999).

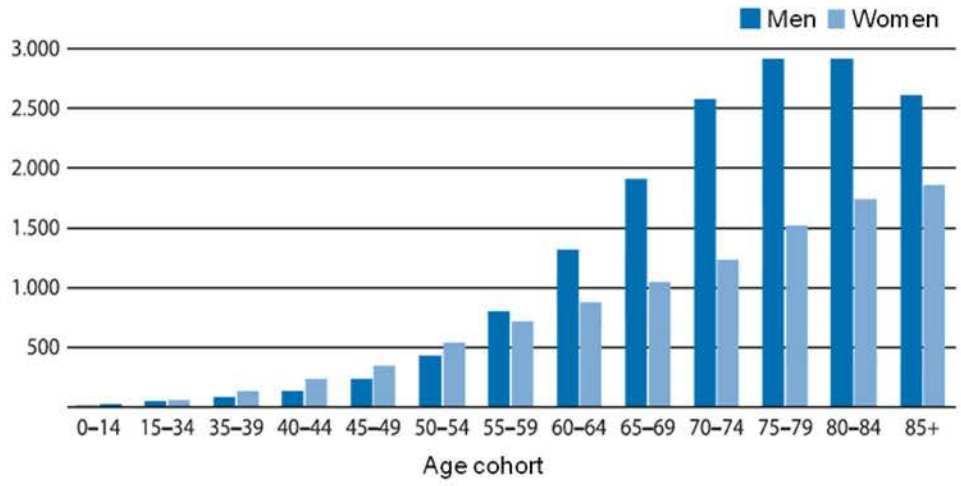


Fig. 7 Incidence of malignant neoplasms in Germany in the year 2004 (Hiddemann et al., 2010, modified)

2.5.1 Fundamentals of carcinogenesis

One of the multiple models of carcinogenesis is the graduation into initiation, promotion, and progression (Schulte-Hermann 1985; Pitot et al., 1989). The initiation describes the development of potential tumour cells, whose morphology is indistinguishable from normal cells. The following development of morphologically recognisable preneoplastic cells by clonal growth is called tumour promotion, and the formation of clinically manifested tumours by further mutation of one of the preneoplastic cells and its selective growth is known as progression. The further transformation of one cell of the benign tumour can lead to malignant lesions (Fig. 8).

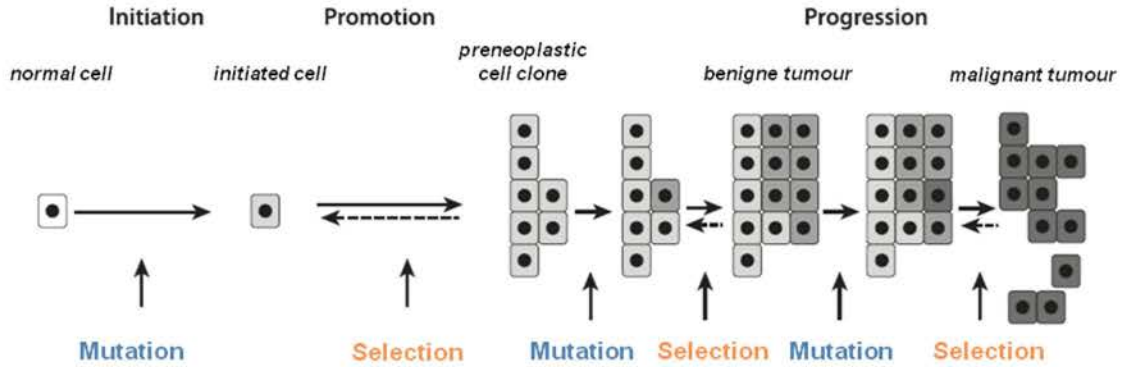


Fig. 8 Multistage process of carcinogenesis
(Schulte-Hermann & Parzefall, 2010, modified) The development of tumour lesions can be divided into initiation, promotion, and progression.

Research over decades has revealed that tumours are more than just masses of proliferating cancer cells; they are complex tissues containing multiple distinct cell types and extracellular matrix (Fig. 9), whose composition has developed with processes comparable to those used by developing organs, although their architecture and functionality is abnormal. This is why tumours cannot be understood just by figuring out the characteristics of the cancer cells, but also by taking into consideration the microenvironment of the tumoral cells (Egeblad et al., 2010; Hanahan & Weinberg, 2011).

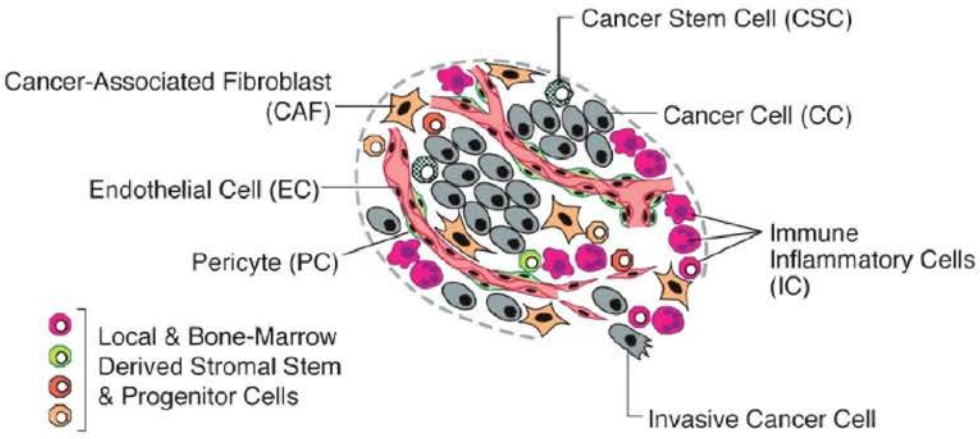


Fig. 9 The cells of the tumour microenvironment

(Hanahan & Weinberg, 2011) Tumours are complex tissues containing multiple distinct cell types and extracellular matrix that collectively enable tumour growth and progression.

In the following table (Tab. 1), a series of noncancerous cells present in tumours and their respective role within tumour development are presented in order to reflect, how versatile the cell composition of a tumour can be (Egeblad et al., 2010, modified).

Tab. 1 Noncancerous cells of the tumour organ and their effects on the tumour (Egeblad et al., 2010, modified)

Cell Type	Effect on Tumors
Normal epithelial cells	inhibit
Myoepithelial cells	inhibit (invasion, growth)
Fibroblasts	promote (proliferation, angiogenesis, invasion)
Mesenchymal stem cells	promote (metastasis)
Adipocytes	promote (tumor growth, survival, angiogenesis)
Endothelial cells	promote (angiogenesis, niche?)
Perivascular cells	promote (vascularization)
Bone marrow-derived cells	promote (proliferation, invasion, angiogenesis)
Dendritic cells	inhibit (stimulate antitumor immunity)
Myeloid-derived suppressor cells and immature myeloid cells	promote (angiogenesis, metastasis,reduce antitumor immunity)
Macrophages, M1-like	inhibit
Macrophages, M2-like	promote (invasion, angiogenesis)
Mast cells	promote (angiogenesis)
Neutrophils, N1	inhibit (stimulate antitumor immunity)
Neutrophils, N2	promote (angiogenesis, reduce antitumor immunity)
T cells, $CD4^+$, T helper 2	promote (metastasis)
T cells, $CD8^+$, cytotoxic	inhibit (tumoricidal)
T cells, $CD4^+CD25^+$ regulatory	promote (reduce antitumor immunity)
T cells, gamma/delta	inhibit (stimulate antitumor immunity)
T cells, Th17	promote (proliferation, angiogenesis) inhibit (stimulate T-cell antitumor immunity)
B cells	promote (reduce antitumor immunity)
B cells, immunoglobulins	promote (stimulate inflammation-associated progression)
Platelets	promote (metastasis)

Cancer cells are known to sustain chronic proliferation, whereas normal tissues maintain their cell homeostasis by carefully controlling the release of growth factors in order to preserve the normal architecture and function of the organ. In normal tissue growth factors bind to cell-surface receptors, which mostly have intracellular tyrosine kinase domains activating the cellular pathways (mitogenic signals) for the regulation

of the cell cycle and cell growth. Theses mitogenic signals further influence cell survival and the energy metabolism. The growth factors control cell number and position in the tissues. In general, they are released by neighbouring cells (paracrine) into the extracellular matrix, where they are activated by a complex network of proteases, sulphatases, and other enzymes (Hanahan & Weinberg, 2011). In contrast, cancer cells deregulate the release of these growth-factors. Some cancer cells exhibit autocrine proliferative signalling by producing the growth factors themselves, or they stimulate other normal cells to supply these (Bhowmick et al., 2004; Cheng et al., 2008, Hanahan & Weinberg, 2011). A further mechanism of cancer cells is the increase of cell receptor proteins to enhance the ligand binding. Moreover, structural alterations of receptors or constitutive activation of proteins in the signalling pathway reveal ligand-independent signalling. Negative feedback loops are also of certain importance: once activated, proteins are inactivated again to stop the signal and avoid sustaining stimulus. A prominent example is the RAS oncoprotein. Replacing GDP by GTP activates the RAS protein, and hence, mutations in the Ras gene deplete the GTPase activity of RAS, resulting in the inhibition of GTP. Consequently, RAS stays in its activated form and accumulates in the cell. The signal is now no longer transitory (Hanahan & Weinberg, 2011).

Recent research revealed that excessive cell proliferation caused by oncoprotein stimulation (such as RAS) can trigger cell death or senescence (Lowe et al. 2004; Evan & d'Adda di Fagagna, 2009; Collado & Serrano, 2010, Hanahan & Weinberg, 2011). Cultured cells receive a nonproliferative, but viable state after expressing high levels of RAS; other cells expressing less of the oncoprotein can avoid senescence and continue proliferation. The mechanism is considered to be a defence mechanism, as cells exhibiting excessive stimulation of a certain signalling pathway are eliminated. Cancer cells either yield a compromise between increased maximal stimulation and antiproliferative defences, or they can adapt to the excessive stimulation by disabling the apoptosis- or senescence features (Hanahan & Weinberg, 2011).

Furthermore, cancer cells can disable negative cell proliferation regulation systems such as tumour suppressor genes. A prominent example is the retinoblastoma-associated protein (RB protein), which responds to various extracellular signals and regulates the proceeding in the cell cycle. Cancer cells with defects in the RB protein

pathway miss an important control function, and persistent cell proliferation occurs (Hanahan & Weinberg, 2011).

Another important mechanism to suppress inappropriate cell proliferation is known as contact inhibition. This phenomenon can easily be observed in two-dimensional cell cultures, with cell proliferation arrest observed as soon as a confluent cell monolayer is built up (Hanahan & Weinberg, 2011). One of the multiple mechanisms related to contact inhibition involves the protein Merlin, which is a gene product of NF2. It connects cell surface molecules such as E-cadherin with receptors like the EGF receptor (a transmembrane tyrosine kinase receptor), and by doing so it first enhances the cell-cell attachments, and second, it disables ligand binding into growth factor receptors (Okada et al., 2005; Curto et al., 2007, Hanahan & Weinberg, 2011). In neoplasia, cells lose the ability of contact inhibition, which can be demonstrated in numerous *in vitro* such as the Cell Transformation Assay. In fact, non-malignant cells grown to confluence in general stop proliferation, but after transformation and a loss of contact inhibition they continue proliferation and form clearly recognisable colonies (Ch.3.3.6) (Hanahan & Weinberg, 2011).

A famous defence mechanism against the transformation of cells into cancer cells is the principle of the programmed cell death by apoptosis. As soon as a cell enters the apoptotic programme, the cell components are packed into vesicles and the cell is decomposed into so called apoptotic bodies. These bodies are either eliminated by neighboured cells or by special phagocytes. There are two major pathways to induce apoptosis: the extrinsic programme in response to extracellular signals and the intrinsic programme triggered by intracellular stimuli. The latter was found to be more widely involved in the physiological cancer defence. Most notable is the identification of DNA damages via the p53 tumour suppressor, which increases the expression of proteins of the Bcl-2 family, dependent on the occurrence of DNA breaks or other chromosomal abnormalities to finally induce the apoptotic programme. A defect in the p53 function means the loss of one of the most important damage sensors. A mechanism to prevent damaged, precancerous cells from elimination is autophagy. The autophagic programme leads to the breakdown and catabolism of cellular molecules and organelles by encompassing them with vesicles (autophagosomes). Following fusion with lysosomes, the content is degraded (Hanahan & Weinberg, 2011). In general, the autophagic programme is induced as a response to cellular

stresses such as nutrient deficiency (Hanahan & Weinberg, 2011), but was also described for the sequestering of toxicants and is believed to be a kind of "first-response reaction" to delay apoptosis (Kundu & Thompson, 2008; Yang et al., 2008). The treatment of cancer cells with cytotoxic drugs might lead to increased autophagy, which is apparently cytoprotective rather than lethal (Hanahan & Weinberg, 2011).

In contrast to apoptosis, necrosis is a cell breakdown, which leads to a blow up of the affected cells, followed by disintegration, and the release of cellular components and proinflammatory signals into the surrounding tissue microenvironment. Hence, inflammatory cells are attracted and activated to induce angiogenesis, cancer cell proliferation, and invasiveness. Moreover, IL-1 α, released by necrotic cells, stimulates neighbour cell proliferation, followed up by neoplastic progression. Consequently, the tolerance of necrotic cell death and the recruiting of inflammatory cells provides an advantage for neoplasias because of growth stimulation to the surviving cells and, as a consequence, tumour promotion (Hanahan & Weinberg, 2011).

For the development of cancer, excessive proliferation requires the interaction with immortalisation. In normal cells chromosomes exhibit non-coding DNA material (telomers) at their ends, which is shortened with every cell division due to limited replication of the intermittent DNA strand. The limitation is based on the fact that the replication can only be processed as long as a primer still can attach to the DNA strand. Hence, small pieces of the DNA strand (up to 100 nucleotides) are lost with every replication. Consequently, the number of cell divisions is limited to the length of the telomers, and the cells finally enter the state of senescence. In cells, where nearly unlimited replication is of importance, such as germ cells and stem cells, an enzyme called telomerase is expressed, which elongates the telomers to protect the cells from chromosomal end-to-end fusion, which results in the destabilisation of chromosomes and a change of the karyotype. The telomerase activity is almost absent in normal cells, but in about 90% of the cancer cells the enzyme is expressed. As a consequence, the cells circumvent senescence and gain the chance to immortalise (Shay & Wright, 2000; Blasco, 2005; Hanahan & Weinberg, 2011).

The induction of angiogenesis in tumour tissue is also one of the most important steps in carcinogenesis, as the tumour will be provided with nutrients and O_2, and can emit metabolic wastes and CO_2. The process of angiogenesis is physiologically

observed in embryogenesis and female reproductive cycling, as well as during wound healing, but always transiently. In tumour progression angiogenesis is almost always observed. A prominent group of angiogenic regulators are the vascular endothelial growth factors (VEGF), whose genes are upregulated by both hypoxia and oncogenic signalling (Hanahan & Weinberg, 2011).

2.5.2 Migration, invasion and metastasis

Tumour metastasis is generally described as a multistep process (Fig. 10). After the primary tumour has been established by cellular transformation, tumour growth, angiogenesis, and the development of a complex tumour microenvironment, local invasion of some cancer cells is observed in malignant tumour tissues.

Especially the thin-walled vessels such as lymphatic channels are easy to penetrate and exhibit only poor resistance for intravastation. Hence, they are the most common entry route for cancer cells into the circulation. The detachment of tumour cells or cell aggregates, respectively, is the next step in metastasis. Nevertheless, most of the circulating tumour cells are rapidly eliminated. The surviving cells get stuck in the capillary system of distant organs and adhere to the capillary endothelium. Extravastation is presumably based on similar mechanisms like invasion and is followed by proliferation and colonisation. A secondary tumour, the metastasis, establishes at the new location by the same mechanisms as utilised in the primary tumour (Fidler, 2003).

To undergo epithelial-mesenchymal transition (EMT), a developmental regulation programme, has been reported as a main tool for transformed epithelial cells to gain the ability of invasion, resistance of apoptosis, and dissemination (Hanahan & Weinberg, 2011). EMT is known as a multifaceted process and can either be transitory or permanent, as well as activated to a different degree during invasion and metastasis. During embryogenesis EMT is initiated and controlled by transcriptional factors such as Snail, Slug, Twist, and Zeb 1/2. Those initiators were observed in various combinations in numerous of malignant tumour types, as well as their mode of action was analysed in cancer models and they were casually associated with invasion and metastasis when overexpressed (Micalizzi et al., 2010; Taube et al., 2010; Schmalhofer et al., 2009; Yang & Weinberg, 2008). Characteristics such as the

loss of adherens junctions, expression of matrix-degrading enzymes, increased motility, and the resistance to apoptosis are common consequences of EMT induced by those transcription factors and are generally associated with the process of invasion and metastasis. Some of these transcription factors for example, can directly suppress E-cadherin, a key cell-to-cell adhesion molecule, and hence, a key suppressor of motility and invasiveness in neoplastic epithelial lesions (Peinado et al., 2004).

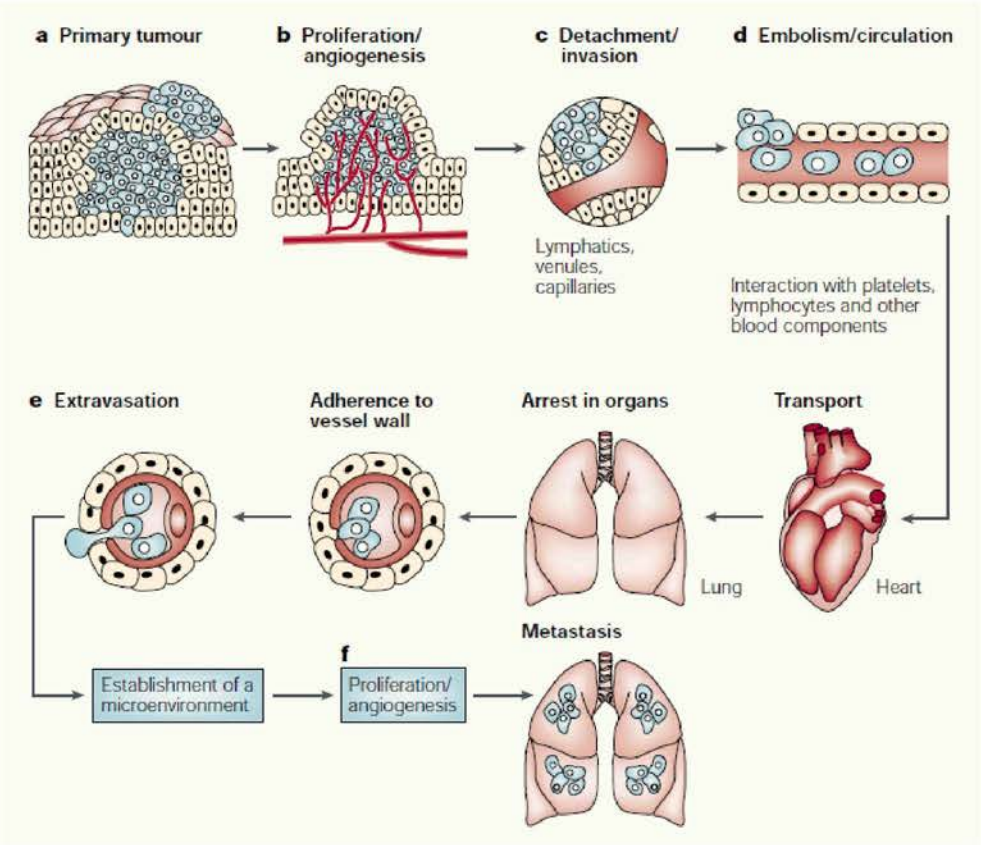


Fig. 10 Invasion Metastasis Cascade
(Fidler, 2003) Tumour metastasis is generally described as a multistep process.

The regulation of the transcription factors included in EMT and their interaction has not yet been fully understood. Research on developmental genetics revealed that also signals are received from neighboured cells, which are involved in the stimulation of those transcription factors. Analogously, it is likely that cancer cells also receive their stimulus for the expression of transcriptional regulators, which subsequently leads to the malignant phenotype, by means of interaction with the cells

of the tumour microenvironment. For example, macrophages can support invasiveness by supplying matrix-degrading enzymes such as metalloproteinases.

Although the question has not yet been fully solved, it is likely that the invasion-metastasis cascade and the following development of secondary tumours is controlled by EMT-inducing transcription factors. Nevertheless, further research has to be done to answer the question, whether invasive cancer cells necessarily have to pass EMT during development of their malignant state, or if alternative processes also lead to this capability.

2.5.3 Cyclooxygenase 2 (COX-2) activation

The COX-2 protein catalyses the biosynthesis of prostaglandines from arachidonic acid, which are known to enhance cell proliferation, angiogenesis, and metastasis, as well as to control apoptosis (Trifan & Hla, 2003). In general, there is no COX-2 activity in the tissues. However, a single stimulus enhances the protein level for only a few hours (Vane et al., 1998). COX-2 is stimulated by factors associated with inflammation, such as bacterial lipopolysaccharide, cytokines, and TNF α. The protein itself further activates inflammatory cells and induces the secretion of growth factors like TGF-ß, which are, among others, important for wound healing and angiogenesis (Vane et al., 1998). COX-2 is known to be an essential factor for the survival of human and mammalian cells under stress conditions, as it protects the cells from stress -induced apoptosis, for example caused by DNA damaging agents (Chai et al., 2007). On the other hand, the induction, expression, and activity of COX-2 is an essential step in the development of colon cancer due to the decrease of apoptosis and the survival of the epithelial cells beyond normal lifetimes, allowing the development of a malignant phenotype. Therefore, COX-2 activity is correlated with the progression of pro-cancerous epihelial cells to fully malignant phenotypes (Vane et al., 1998). The pro-carcinogenic property of COX-2 was demonstrated in experimental animal models, showing that inhibitors of COX-2 reduced the formation of intestinal, breast, skin, lung, bladder, and tongue tumours (Vainio, 2001). In malignant tissues from colorectal cancer and polyp tissues from patients, as well as in human gastric and breast tumours, COX-2 protein levels were elevated as compared to the surrounding tissue (Vane et al., 1998). In Apc mice, which serve as

a model for human familial adenomatous polyposis, the deletion of the COX-2 gene (official gene name PTGS2) or the treatment with a selective COX-2 inhibitor led to decreased intestinal polyposis. Rat epithelial cells infected with cDNA of COX-2 showed consequently increased prostaglandine synthesis and decreased apoptosis rates (Vane et al., 1998). Xiong et al. (2005) determined the clinicopathologic significance of COX-2 expression in human colorectal cancer. They reported that 67.9% of the colorectal cancer cases were detected positive for COX-2 protein when they were immunohistochemically analysed with anti-COX-2 antibodies. 86.7% of the cases with lymphnode metastasis had elevated COX-2 levels in the primary lesions, and 100% of these patients were analysed positive for COX-2 in the cancer cells of the metastatic lesions. Furthermore, the positive expressions of VEGF and MMP-2 were closely correlated with that of COX-2. This is the reason why the latter is discussed to alter the invasive potential of colorectal cancer cells through the activation of MMP-2. Hence, COX-2 is associated with tumour progression by modulating angiogenesis, cancer cell motility, and the invasive potential in colorectal cancer. Phenotypic changes of rat intestinal epithelial cells, including increased adhesion to extracellular matrix, decreased apoptosis, and enhanced tumourigenic potential, were related to COX-2 overexpression (Xiong et al., 2005). Endothelial cell migration and vessel tube formation, as well as activated collagenase levels induced by COX-2 were reported by Masferrer et al. (2000). Because of the significant correlation of COX-2 with the depth of invasion, the state of disease, and the occurrence of metastasis, COX-2 is suggested to be a possible biomarker (Xiong et al., 2005).

2.5.4 The role of miRNAs in carcinogenesis

MicroRNAs (miRNAs) are about 22 nucleotide long RNA molecules with regulatory function. There are more than 500 miRNA genes known so far. In the nucleus miRNA genes are first transcribed into several hundred base pair (bp) long RNAs containing a hairpin structure (pri-miRNA), which are then further processed to about 70 bp long pre-miRNAs (Fig. 11).

The processing is executed by the RNases III Drosha and Pasha. The prä-miRNAs are then excreted into the cytoplasm, where the RNases Dicer and Loqs transform

them into 22 bp long duplices. Only one strand of the duplices can be incorporated into a large protein complex (RNA -induced silencing complex, RISC) and guide it to its complementary target segment of the mRNAs. The aim is to regulate the target mRNA either by blocking further translation, or by its degradation. It is estimated that at least 30% of all human genes are regulated by miRNAs, and one miRNA can regulate the expression of up to 1,000 genes by either direct interaction with the respective mRNA, or by further indirect mechanisms: so called epi-miRNAs were recently found to regulate epigenetic enzymes, such as DNA methyltransferases and histone deacetylases (Bartram, 2010; Zimbardi et al., 2012).

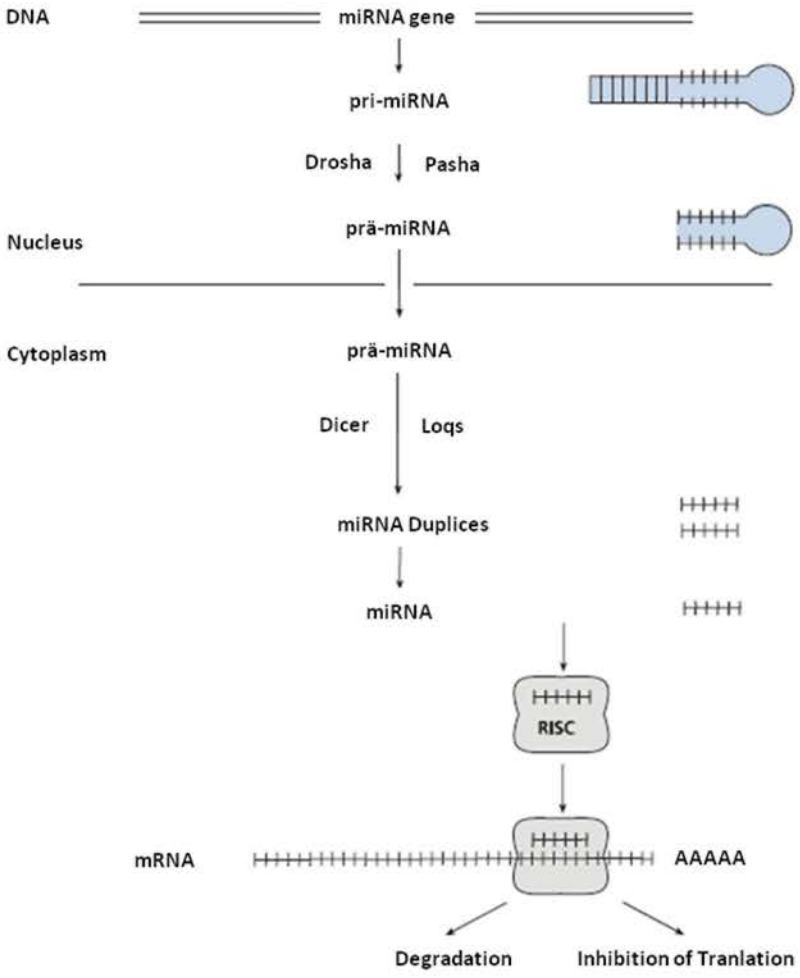


Fig. 11 miRNA sythesis und function
(Bartram, 2010, modified)

In tumour tissue the regulation via miRNAs is of great relevance. Among classically known cancer genes miRNAs are known as molecular switches. They were described as essential components of fundamental signal transduction of carcinogenesis (Dalmay & Edwards, 2006; Sotiropoulou et al., 2009). Comparable to protein-coding genes, miRNAs can also be downregulated by DNA hypermethylation of the promoter regions in cancer cells (Zimbardi et al., 2012).

Altered miRNA profiles in bladder cancer tissues were recently reported (Catto et al., 2009; Dyrskjot et al., 2009; Ichimi et al., 2009; Lin et al., 2009; Veerla et al., 2009; Hanke et al., 2010); among them miR-200a (Lin et al., 2009) and miR-205 (Dyrskjot et al., 2009; Gottardo et al., 2007). miR-139 was detected to be decreased in bladder tumours (Ichimi et al., 2009), possibly targeting CXCR4 and resulting in metastasis. CXCR4 was described to be increased in invasive bladder tumours (Retz et al. 2005; Dalmay & Edwards 2006). Gregory et al. (2008) described miR-200-family (miR-200a, -200b, -200c, -141, and -429) in combination with miR-205 regulating epithelial-mesenchymal transition (EMT). EMT is known to be closely correlated with invasion and migration of tumour cells, as well as metastasis of tumours. MiR-200-miRNAs inhibit the expression of the proteins Zeb 1 and Zeb 2, which suppress the transcription of CDH1. Decreased levels of miR-200-miRNAs are correlated with progression in breast cancer and reveal poor prognosis for ovarian carcinoma (Gregory et al., 2008; Hu et al., 2009). miR-146a was detected to play a role in suppression of metastasis in breast cancer (Hurst et al., 2009), among others, by decreasing protein levels of MMP9, which is important for the invasion of tumour cells (Bhaumik et al., 2008). ROCK1 was identified to be a target of miR-146a in prostate carcinoma cell lines, as well as CXCR4 in leukaemia cells (Labbaye et al., 2008; Lin et al., 2008). Nevertheless, care has to be taken when the relevance of altered miRNA profiles is interpreted in carcinogenesis, as their expression patterns are highly specific for cell-type and cellular differentiation status. Hence, it is likely that altered expression patterns are not conclusively the reason for malignant transformation, but a secondary consequence of the loss of the normal cell identity accompanied by malignant transformation (Kent & Mendell, 2006).

2.5.5 DNA methylation

The genetic information flow is not only dependent on the primary DNA sequence, but also on the fact that the functional structures of the transcriptional system, such as the RNA-polymerase II, gain access to the relevant gene loci. Specific modifications of the DNA sequence and the surrounding chromatin regulate genetic programs by either inhibition or activation. These modifications are tissue specific and dependent on the developmental stage. Hence, they are inherited to the following cell generations. This regulatory system is generally reversible and independent from the primary DNA sequence. Modifications in the so-called epigenetic system can reveal consequences of similar concern like mutations in the genetic information itself (Bartram, 2010; Zimbardi et al., 2012).

DNA methylation is generally performed by DNA methyltransferases (DNMTs), which transfer a methyl group from S-adenosyl methionine (SAM) to the cytosine nucleotide (Ren et al., 2011). Hereby, the most relevant modification is the methylation of cytosine at CpG sites. These are dinucleotides consisting of a cytosine and a guanine in direct neighbourhood. In CpG islands these dinucleotides are found at higher frequencies, and about 37% of the approximately 38,000 CpG islands can be found in the promoter region of about 50% of the genes. Their methylation leads to the silencing of the respective gene (Bartram, 2010). Hence, DNA methylation patterns are a crucial part of the epigenetic gene regulation to maintain the normal homeostasis of gene expression (Zeimet et al., 2010).

De novo methylation is catalysed by the DNA methyltransferases DNMT3A and DNMT3B. Both have similar functions but also different tissue and DNA-target structure specifications (Reik et al., 1999). The DNA methyltransferase DNMT1 organises the maintenance of the DNA methylation patterns in the context of cell proliferation and modifies the daughter strands to avoid passive demethylation.

Following methylation, methyl-CpG-binding domain proteins (e.g. MeCP2) are associated and mask the recognition sequence of transcription factors. Hence, the transcriptional activation system is blocked (Bartram, 2010).

Global DNA hypomethylation is correlated with chromosomal instability, like it is observed for example in colon carcinoma (Matsuzaki et al., 2005).The majority of hypomethylation occurs at repetitive elements such as long interspersed nuclear elements (LINEs). There are about 500,000 copies of LINE1, and most of them have

lost their functionality during human evolution. However, genome wide hypomethylation of LINE1 may initiate the clonal expansion of premalignant cells in early stages of cancer development, is presumed to contribute crucially to chromosomal instability, and may lead to structural changes in chromosomes (Wilhelm et al., 2010; Zeimet et al., 2010). Choi et al. (2009) reported that LINE1 methylation was very consistent from individual to individual and from tissue to tissue. There was a clear hypomethylation of LINE1 in bladder cancer, but not in leukaemia, leading to the assumption that DNA methylation of repetitive elements such as LINE1 can serve as biomarkers.

Oncogene activation due to DNA hypomethylation is rarely reported (Ehrlich, 2002). One example is the abnormal gene expression of BCL2 in patients suffering from B-cell chronic lymphocytic leukaemia (Hanada et al., 1993). In contrast, there are numerous tumour suppressor genes inactivated by DNA hypermethylation in various kinds of tumours (Bartram, 2010). One prominent example of misleading epigenetic regulation is related to the P16 protein, which is a cyclin-dependent kinase (CDK) inhibitor. P16 is known to block the cyclin-D mediated activation of the protein kinases CDK4/6 and enable the retinoblastoma protein (RB) to prevent the cell from replicating damaged DNA by preventing its progression along the cell cycle through the first gap phase G1 into the synthesis phase S. Most of the tumours in man exhibit disruptions in this signal transduction pathway, because of the functional loss of either P16 or RB (Sherr 1996; Baylin et al. 1998). Notably, the loss of function of P16 can be based on both, gene deletion or mutation, as well as on promoter hypermethylation, whose frequency varies between the different tumour forms. In colon carcinomas hypermethylation is the major mode of action for the inactivation of P16 (Bartram, 2010).

Promoter hypermethylation of the gene coding for the cell adhesion molecule E-cadherin (*CDH1*), is often found in epithelial tumours and results in the loss of inhibition of cell invasion (Yoshiura et al., 1995; Conacci-Sorrell et al., 2002). Nevertheless, Chim et al. (2003) reported only poor hypermethylation of *CDH1* in patients suffering from acute promyelocytic leukaemia, in contrast to acute myeloid leukaemia. In bladder specimens *CDH1* was reported to have similar methylation patterns in both benign and malignant tumours and was found to be no suitable biomarker (Pu et al., 2006).

A further prominent gene in tumourigenesis is the retinoic acid receptor, beta gene, RARB, which codes the retinoic acid receptor ß (RAR ß). Retionids are vitamin A derivates with effects on growth control, epithelial differentiation, and embryonic development. There are three isoforms of RAR (α, ß, γ), which all are nuclear receptors and transcription factors. They bind to the promoter of their target genes as heterodimers together with retinoid X receptors (RXR) and repress or activate the transcription depending on the presence or absence of their ligand hormone. Both retinoic acid and the three RARs are closely linked to cancer and are known to have tumour suppressive properties. Several alterations in the expression of RAR ß were reported for various kinds of tumours. The major mode of action is believed to be the loss of repressive function on the activator protein 1 (AP-1), which itself controls a number of cellular processes including differentiation, proliferation, and apoptosis (Lei & Thé, 2003). In bladder cancer, altered methylation of RARB was reported in several studies (Chan et al., 2002; Hoque et al., 2006, Negraes et al., 2008).

In lung and breast cancer Ras Association Domain family 1 gene (RASSF1) was detected to undergo epigenetic inactivation by hypermethylation of the promoter region. There are two major transcripts, RASSF1A and RASSF1C, which are produced by alternative promoter selection and mRNA splicing. RASSF1A was reported to be a potential tumour suppressor gene. It is known to inhibit proliferation by negatively regulating cell cycle progression through G1 into S phase and inducing cell death as a negative Ras effector (Dammann et al., 2005; Burbee et al., 2011). Promoter hypermethylation of RASSF1A was detected in more than 50% of the bladder cancer cases analysed, but in none of the controls. Furthermore, RASSF1A was associated with invasive bladder cancer, the more advanced and fatal form of this disease (Dulaimi et al., 2004; Dammann et al., 2005).

In recent scientific publications C1qTNF6, a protein with an unique molecular structure including, among others, a collagen-like domain, has come into focus. Initially, this protein was reported to be involved in fatty acid metabolism and to play a role in the energy balance (Lee et al., 2010). Additionally, C1qTNF6 was reported to induce the expression of IL10 and thus, be a potential pharmacological target in inflammatory diseases (Kim et al., 2010). Lately, the expression of C1qTNF6 was detected in hepatocellular cancer, and moreover, its possible support of tumour angiogenesis in this cancer type was discussed (Takeuchi et al., 2011). This is in

accordance with the findings of Wong et al. (2008), who reported endocrine actions of C1qTNF6. However, the pathologic role of this protein and its expression status in carcinogenesis still remains unclear and is of further interest. Up to now no information is available on the epigenetic regulation of the protein expression.

The inhibition of DNA repair by promoter methylation of genes coding for DNA repair proteins is another well-known mechanism in tumourigenesis. One prominent example is O^6-methylguanine-DNA methyltransferase (MGMT), which transfers the alkyl groups from the O^6-position of guanine onto itself. This protein is not a true enzyme, since the protein is not regenerated after the reaction but degraded. Aberrant MGMT promoter methylation leads to aberrant protein amounts and decreased defence against genotoxicity. Many immortalised and tumour cell lines lack this protein; in contrast, it was detected in primary tumour samples. The levels of this protein vary in human tumoral and normal tissues. Nevertheless, some studies presume a correlation between MGMT methylation status and the susceptibility of tumour development (Ekim et al., 2011; Pegg, 2011).

Inactivation of apoptotic factors by aberrant promoter hypermethylation was also observed in cancers such as therapy resistant melanomas, where the inactivated apoptotic protease activating factor - 1 (Apaf-1) was detected. Aberrant methylation of tumour suppressor genes can also be of prognostic relevance, as it was reported for the acute lymphoblastic leukaemia: the more genes are affected by aberrant methylation, the poorer is the prognosis (Bartram, 2010).

Finally, misleading epigenetic regulation of miRNAs can also lead to tumorigenesis. Hypermethylation of miR-124a was observed in various neoplasia and was correlated with the activation of CDK6 and the repression of RB (Lujambio et al., 2007).

There are numerous further genes, whose altered methylation patterns were described in bladder tumours that were suggested as possible biomarkers. But as cancer development is a complex process, only analysis of a panel of multiple genes could lead to valuable results in non-invasive cancer diagnosis (Zimbardi et al., 2012).

2.5.6 Arsenic-induced carcinogenesis

Chronic arsenic exposure is associated with an increased risk of cancer. Especially inorganic arsenic is a well known group I carcinogen recognised by the International Agency for Research on Cancer (IRAC) and the World Health Organization (WHO) (Zhang et al., 2007; Ren et al., 2011). Arsenic-induced lung cancer, as well as skin, kidney, and bladder cancer were reported (Chiou et al., 1995; Tseng, 2007; Chen et al., 2010; Ren et al., 2011). A detailed report on an epidemiological study over 50 years showed that in a Chilean population a significant increase in bladder cancer mortality was correlated with higher arsenic concentrations in drinking water (Marshall et al., 2007). Moreover, indications are given that the biological behaviour of urothelial carcinomas in areas highly contaminated with arsenic, such as the southwest coast of Taiwan, are more aggressive than urothelial carcinomas from a non-contaminated area (Chen et al., 2007). As arsenic is metabolised after uptake, research has also been focussed on the toxicity and carcinogenicity of the metabolites. Especially the trivalent arsenicals are known to exhibit strong genotoxic effects (Dopp et al., 2010a). Furthermore, studies in rats have shown that ingestion of DMA(V) causes bladder cancer after chronic exposure (Wei et al., 1999).

One generally accepted mechanism for arsenic toxicity and presumably also arsenic-induced carcinogenesis is the generation of reactive oxygen species (ROS). While ROS physiologically occur during cellular respiration or aerobic metabolism they also can result from exposure to oxidants. Already low levels of As(III) and MMA(III) are reported to generate ROS and therefore cause oxidative stress (Eblin et al., 2008; Wnek et al., 2009, as cited in Zdrenka et al., 2012[3]). These highly reactive radicals are discussed to exhibit their toxicity via induction of DNA damage, formation of DNA adducts, or alteration of DNA methylation and histone modifications (Wnek et al., 2009), finally leading to carcinogenesis (Kitchin & Ahmad, 2003; Huang et al., 2004, as cited in Zdrenka et al., 2012). In addition, arsenic is known to interfere with nucleotide and base excision repair at very low, non-cytotoxic concentrations and was observed for both trivalent and pentavalent metabolites. Hereby, MMA(III) and DMA(III) were found to exhibit the strongest effects (Hartwig et al., 2003, as cited in Zdrenka et al., 2012). One key mechanism is the inactivation of poly (ADP-ribose)

[3] Available information on the mechanisms discussed for arsenic-induced carcinogenesis was recently reviewd by Zdrenka et al. (2012). The respective information was interspersed into this chapter.

polymerase (PARP) already at extremely low, environmentally relevant concentrations (Hartwig et al., 2002; Hartwig et al., 2003, as cited in Zdrenka et al., 2012). Wnek et al. (2009) reported that the relative PARP activity was significantly reduced during chronic exposure of immortalised human urothelial cells (UROtsa) to 50 nM MMA(III). After removal of MMA(III) PARP activity increased again. Trivalent arsenic species are known to attach to zinc-binding structures generally found in DNA repair enzymes, leading to alteration or inhibition of these proteins and finally the loss of genomic integrity (Kitchin & Wallace, 2008, as cited in Zdrenka et al., 2012). In contrast, former studies report the insensitivity of isolated and purified DNA repair enzymes against inhibition by arsenic (Hu et al., 1998, as cited in Zdrenka et al., 2012). This leads to the assumption that there are different modes of action for arsenic inhibited DNA repair, on the one hand by directly targeting DNA repair proteins, and on the other hand by altering signal transduction or gene expression (Zdrenka et al., 2012).

Another mechanism of arsenic-induced carcinogenesis is the altered cytoplasmic and nuclear signal transduction, modifying proteins involved in cell proliferation, differentiation, and apoptosis (Wnek et al., 2009, as cited in Zdrenka et al., 2012). Hereby, ROS were detected to be one key mechanism for the influence of As(III) and MMA(III) on the mitogen-activated protein kinase (MAPK) signalling pathway leading to consistent changes in cellular signalling (Eblin et al., 2008, as cited in Zdrenka et al., 2012). The persistence of MMA(III)-induced altered cellular functions even after the termination of arsenic exposure point to lasting genomic or epigenetic changes, and thus the highly carcinogenic potential of arsenic and its metabolites (Zdrenka et al., 2012).

Elevated mRNA levels of COX-2 were detected in arsenite exposed normal human epidermal keratinocytes at concentrations comparable to arsenic exposed populations (Hamadeh et al., 2002). In addition, chronic exposure to 50 nM MMA(III) for 12, 24, and 52 weeks led to time dependent increases of COX-2 and epidermal-growth-factor-receptor (EGFR) activation (Eblin et al., 2009), which was correlated with phenotypic alterations and the development of malignancy. The authors also report the induction of COX-2 via the mitogen-activated protein kinase (MAPK) signalling, which is activated by the increase of reactive oxygen species (ROS). Previous studies showed increased COX-2 protein levels and increased intracellular

ROS in UROtsa cells exposed to MMA(III) for 52 weeks (Eblin et al., 2007). Both studies report the increased steady state mRNA of COX-2. In contrast, exposure to 1 µM As(III) for 52 weeks led to statistically significant decreased COX-2 protein levels. The authors concluded that MMA(III) has more influence on the MAPK signalling than As(III) (Eblin et al., 2009). The authors reported that COX-2 is dependent on the continuous generation of ROS, as ROS inhibition resulted in a decrease of COX-2 in a comparable manner as with a specific COX-2 inhibitor. Furthermore, depletion of ROS in UROtsa cells treated with MMA(III) for 52 weeks led to colony formation results similar to those of non-treated UROtsa cells, whereas MMA(III)-treated UROtsa cells without ROS depletion showed stronger tumourigenity by an increased colony formation in soft agar. Based on the fact that COX-2 is important for a sustaining anchorage-independent growth, the protein was concluded to be required for the arsenic-induced transformation process (Eblin et al., 2009).

A connection between arsenic-induced carcinogenesis and altered DNA methylation patterns is currently discussed, because both, arsenic biomethylation and DNA methylation, utilise the same methyl donor SAM, which is also involved in many further metabolic reactions. As SAM is the unique methyl donor in arsenic metabolism, it is proposed that chronic arsenic exposure might deplete SAM, especially in combination with methionin insufficient diet leading to decreased SAM synthesis. Many studies report hypomethylation after arsenite exposure both *in vitro* and *in vivo.* Reichard et al. (2006) report a depletion of SAM in human HaCaT cells after treatment with sub- to low-micro molar concentrations of arsenite. In addition, arsenite repressed the expression of DNMT1 and DNMT3A. Based on these results the authors conclude that long-term exposure to arsenite might lead to DNA hypomethylation. Moreover, only very few data for the human system report that arsenic exposure, e.g. via contaminated drinking water, leads to global DNA hypomethylation (Ren et al., 2011). Especially, the hypomethylation of LINE1 was observed in subjects suffering from bladder cancer. A strong correlation between the risk of non-invasive bladder cancer and LINE1 hypomethylation was observed, but in contrast, there was no significant association to invasive bladder cancer cases. The authors suggested that LINE1 hypomethylation is an independent risk factor for bladder cancer and might serve as an excellent biomarker. However, a dose-dependency between LINE1 methylation extent and the risk of bladder cancer was

not observed (Wilhelm et al., 2010). Further studies have to be performed to assay whether LINE1 methylation patterns are directly targeted by arsenic.

Among global DNA hypomethylation, promoter hypermethylation was also observed in human skin and bladder cancer, in animals *in vivo*, as well as in human and animal cell lines *in vitro* (Ren et al., 2011). In patients suffering from bladder cancer after arsenic exposure, promoter hypermethylation of RASSF1A was observed. The excess of arsenic incorporation was measured from toenail arsenic content, and the authors detected a correlation between arsenic exposure, RASSF1A promoter methylation, and bladder cancer occurrence (Marsit et al., 2006b). Moreover, in A/J mice arsenic exposure resulted in promoter hypermethylation of RASSF1A and was associated with the development of lung cancer (Ren et al., 2011).

Only very poor information is available on the effect of arsenic treatment on miRNA profiles, especially in bladder cancer. Marsit et al. (2006a) detected generally increased miRNA profiles after arsenic exposure in human lymphoblastoid cells. The results were similar to those received from cells cultured under folate deficiency, which is known to result in altered SAM levels and altered global genomic methylation. Both, exposure to arsenic as well as folate deficiency, induced significantly different miRNA profiles than those from cells grown under normal control conditions, where arsenic-induced a less dramatic increase as compared to folate deficiency. In detail, hsa-miRNA-22, hsa-miRNA-34a, hsa-miRNA-221, hsa-miRNA-222,and hsa-miRNA-210 were affected by both treatments. Importantly, the latter miRNA was the only one whose expression was decreased. Due to similar effects of arsenic exposure and folate deficiency the authors conclude that altered miRNA expression is based on SAM depletion and, as a consequence, changes in DNA methylation patterns. Hence, arsenic is believed to operate by altering the one-carbon metabolism and thus exhibit downstream epigenetic effects. Aberrant phenotypes during tumourigenesis were suggested to result from alterations in key miRNA expression at critical points in development. Nevertheless, the induced changes in miRNA expression were not stable and returned to baseline levels upon removal of stress conditions, suggesting that chronic exposure may be necessary to permanently alter the expression of miRNAs.

There are few data available concerning arsenic-induced angiogenesis. Kao et al. (2003) reported bilateral effects of arsenic exposure concerning angiogenesis *in vitro*.

0.1 µM sodium arsenite significantly increased tube lengths of Human Umbilical Vein Endothelial Cells (HUVEC) seeded on Matrigel (1.54 fold as compared to control), whereas at concentrations of 5 µM and above tubing integrity was disrupted. Furthermore, sodium arsenite treatment of HUVEC cells induced expression of $VEGF_{121}$ by 2.8 fold as compared to control at concentration of no more than 0.1 µM. Similar results were obtained after As_2O_3 treatment in HeLa cells at high concentrations of 5 and 10 µM, which indicated on a dose dependent inhibition of CD44v6, VEGF-C, and MMP-9 mRNA synthesis (Tab. 2; Du et al., 2009).

Tab. 2 Results of the semi-quantitative RT-PCR analysis in HeLa cells after 48 h exposure to As_2O_3
(Du et al., 2009, modified)

Gene	untreated control	5 µM As_2O_3	10 µM As_2O_3
CD44v6	0.386 ± 0.049	0.244 ± 0.120	0.177 ± 0.030
VEGF-C	0.605 ± 0.166	0.344 ± 0.038	0.150 ± 0.036
MMP-9	0.966 ± 0.205	0.466 ± 0.121	0.220 ± 0.098

These findings are further supported by data from Soucy et al. (2003). As(III) induced a dose dependent increase in vessel density in the chicken chorioallantoic-membrane (CAM) assay, but at concentrations above 1 µM inhibition of vessel growth was observed. The authors concluded a threshold concentration of 0.33 µM arsenite for arsenic-induced angiogenesis in the CAM assay. Taken together, *in vitro* assays gave strong indications for arsenic-induced angiogenesis at low, environmentally relevant concentrations. As angiogenesis is a very important step in the development of solid tumours, this data is very concerning. Further *in vivo* data are needed to determine, whether arsenic exhibits a direct influence on angiogenesis in arsenic-induced cancer diseases, or if this is just a secondary effect during carcinogenesis.

Among molecular markers of arsenic-induced tumourigenesis also the resulting malignant phenotypical changes *in vitro* were assayed. Wen et al. (2008) observed increased population doubling times, enhanced motility and invasiveness, as well as the development of anchorage-independent growth of immortalised human small airway epithelial cells (SAEC) after subchronic treatment with 0.5 µg/mL sodium

arsenite for 6 months. These findings were furthermore correlated with an increase in micronuclei formation and the increased expression of oncoproteines such as c-myc, c-Ha-ras, and c-fos. The authors conclude that long-term arsenite treatment has caused an oncogenic cell transformation. These findings are supported by the results obtained in mouse skin epidermal cells JB6 Cl14, which developed anchorage-independent growth after arsenic treatment (Huang et al., 1999). More recent studies with UROtsa cells came to comparable results after chronic low-dose transformation with arsenic. Interestingly, both As(III) and MMA(III) caused increased anchorage-independent growth after up to 52 weeks of exposure (Bredfeldt et al., 2006; Jensen et al., 2009). Moreover, xenografts of UROtsa cells treated with 50 nM As(III) for 52 weeks into SCID mice resulted in significantly increased subcutaneous tumour formation as compared to the untreated control (Bredfeldt et al., 2006). Furthermore, already 12 weeks of exposure to 50 nM MMA(III) were sufficient to cause enhanced tumourigenesis after injection (Wnek et al., 2010). Similar results were observed in earlier studies. Low-dose arsenite (0.5 µM) induced malignant transformation of rat liver epithelial cells TRL 1215 and led to metastatic tumour growth after injection into nude mice (Zhao et al., 1997).

In summary, all these studies revealed that carcinogenesis in general, but especially arsenic-induced (bladder) cancer has multifaceted modes of action. Despite cytotoxic effects of arsenic treatment at concentrations of 1 µM and above, environmentally relevant low doses revealed cytotoxic tolerance in combination with diverse cellular effects such as genotoxicity and epigenetic modifications, leading to e.g. inhibited DNA repair, altered cell cycle progression, and morphological changes. In addition, not only inorganic arsenic itself, but also its metabolites originating from biomethylation processes, exhibit tumourigenic effects. Further studies are urgently needed to connect those manifold results and develop a complete model of arsenic-induced (bladder) cancer. Only a better understanding of these mechanisms undergone during arsenic-induced (bladder) carcinogenesis can be the basis for more effective diagnostic methods at earlier disease stages by utilising biomarkers, finally leading to a more successful medical treatment.

2.6 Aim of the present work

The aim of the present work was to contribute to a better understanding of the mechanisms behind arsenic-induced cancer, especially regarding bladder cancer. For this purpose, a series of studies was undertaken, to correlate arsenic metabolism and genotypic effects with epigenetic modifications and phenotypical alterations under chronic exposure conditions *in vitro*:

1. The cellular uptake and intracellular biotransformation of arsenic species in non-methylating human urothelial cells (T24) in comparison to methylating human hepatic cells (HepG2) was investigated, and the detected intracellular arsenic was speciated and quantified.

2. Induced genotoxic effects in T24 cells were measured with the Alkaline Comet Assay. To investigate the impact of metabolic capacitiy of the affected tissue on the extend of genotoxicity, both non-methylating T24 cells and methylating HepG2 cells served as a cellular model. Furthermore, primary human uroepithelial cells (HUEPC) were utilised in this test to estimate differences in susceptibility against genotoxicity of primary and permanent cell culture systems.

3. MiRNA analysis and examination of altered DNA methylation patterns were carried out in T24 cells to determine the arsenic-induced modulation of epigenetic regulation systems. Additionally, COX-2 protein, an important molecular markers, was analysed. The tests were carried out throughout the course of chronic low dose treatment with arsenic and aimed to investigate a possible time dependency.

4. The malignant transformation following chronic arsenic treatment was assayed using the Mammalian Cell Transfomation Assay, the Colony Formation Assay, and the Migration and Invasion Assay. The tests were carried out to investigate the influence of arsenic on a malignant phenotype and addressed endpoints such as loss of growth inhibition and the acquirement of three-dimensional growth abilities and enhanced motility, as well as invasive properties.

Since the various arsenic species emerging from the hepatic biotransformation of arsenic exhibit distinctly different toxicity, this study focussed especially on effects induced by methylated metabolites such as MMA(III).

The study was conducted to enhance the understanding of the mechanisms behind arsenic-induced toxicity and carcinogenesis in the urinary bladder epithelium.

3 Material and Methods

For detailed information on applied chemicals and solutions please refer to Annex 7.1.

3.1 Cells and culture conditions

3.1.1 Human urothelial cells: UROtsa and T24 cells

The UROtsa cell line was immortalised by introduction of the SV40 large T-antigen into human urothelial cells (Petzold et al., 1995, Johnen et al, 2013), thus having a stable karyotype without developing anchorage-independent growth properties in later passages, as compared to cells immrotalised with the live SV40 (Christian et al., 1987, Johnen et al,. 2013). UROtsa cells were further described to be non-tumorous in immuno-compromised mice, therfore serve as a model for normal human urothelial cells and are considered to be a suitable test sytem to explore the mechanisms of bladder carcinogenesis *in vitro* (Rossi et al., 2001; Eblin et al., 2008; Johnen et al., 2013). When maintained in serum-coontaining cell culture medium, UROtsa cells are relatively undifferentiated and only form monolayers instead of stratified layers like those observed from primary cells. In contrast, when maintained under serum-free conditions, UROtsa cells were reported to form structures comparable to the intermediate layer of the bladder epithelium *in vivo* (Rossi et al., 2001; Johnen et al., 2013).

UROtsa cells used in the present study were a generous gift from Prof. M. Styblo (University of North Dakota, USA). However, experiments conducted in this laboratory after the present study revealed uncertanities about the identity of the UROtsa cells used. A full analysis to investigate this question was recently performed by G. Johnen and colleagues (Institute for Prevention and Occupational Medicine of the German Social Accident Insurance, Institute of the Ruhr-University Bochhum, Germany). The data exhibited that the UROtsa cells used in this laboratory actualy are T24 cells (transitional cell carcinoma of the urinary bladder), which was proven by means of RNA expression, DNA methylation, and genomic analyses (short tandem repeat (STR) profiling). It is assumed that cross-contamination might have occurred

already before 2004, however, the time and place of the contamination could not be determined (Johnen et al., 2013).

As early as 1973 the T24 cell line was derived from an invasive human transitional cell carcinoma from the bladder of a female patient (Gildea et al., 2000). The cell line was characterised by its non-tumourigenicity when injected into SCID mice. However, it became apparent that over time variants of the T24 cell line have established exhibiting evidently higher tumourigenicity than the original cell line, which is why one of these was named T24T (Gildea et al., 2000). The authors investigated a study to characterise the differences between T24 and T24T cells, showing that in contrast to T24, the latter cell line lost the property of contact inhibition (as indicated by means of the Colony Formation Assay and the *in vitro* Mammalian Cell Transformation Test), are more motile at higher cell densities in the cell migration assay, and form tumours when injected into SCID mice. On the other hand, both cell lines showed the ability to degrade matrigel, which simulates extracellular matrix, and were therefore proven to exhibit proteolytic activities. Observations made in the present study indicate that the cell line contaminating the UROtsa stocks actually is the non-tumourigenic T24 cell line rather than the tumourigenic T24T cell line.

T24 cells contaminating the UROtsa stocks showed varying chromosome numbers, but only relatively moderate alterations of gene expression and gene methylation paterns during long-term culturing. The data show that our cells slightly differ from the ACC376 T24 cells registered at the STR database of the German Collection of Microorganisms and Cell Cultures (DSMZ) and the HBT-4 T24 cells distributed by the American Type Culture Collection (ATCC) (Johnen et al., 2013): the STR analysis of our T24 cells was 100% identical with the HBT-4 data of the DSMZ database and differed in only one allel from the ACC376 serving as concurrent control for the analyses. Among this, DNA methylation patterns of AC374 and our T24 cells are similar but not identical. The karyotype reported for all three T24 stocks is slightly different: ATTC data show 86 chromosomes for HBT-4 cells, the DSMZ reports 70-77 chromosomes, and our T24 cell line exhibited varying chromosome numbers between 55 and 83. Whether our T24 cell line evolved so far from the known T24 stocks that it could be considered to represent a new variant could not be confirmed so far (Johnen et al., 2013). Therefore, the cell line used in our laboratory will in the following only be named as T24 cell line.

The T24 cells were maintained in Earle's minimal essential medium (MEM) enriched with 10% foetal bovine serum (FBS), 0.5% Gentamycin, and 1% L - glutamine. They were incubated at 37 °C and 5% CO_2 atmosphere in 75 cm² flasks. The T24 cells were observed to grow adherent and in a monolayer (Fig. 12) (Zdrenka et al., 2012).

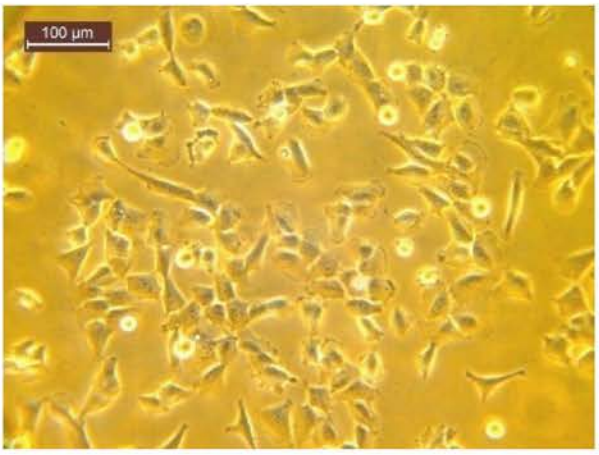


Fig. 12 T24 cells grown as a monolayer in a 75 cm² flask

3.1.2 Human urothelial epithelial cells HUEPC

Human urothelial epithelial cells (HUEPC) (Fig. 13) were obtained from Provitro (Cat.-No.: 1210721; Provitro GmbH, Berlin, Germany). They were produced on the base of human tissues and isolated according to known procedures (Provitro GmbH, 2012). The primary cells were maintained in the serum-free urothelial cell growth medium supplemented with < 0.5% BPE (bovine pituitary extract), < 0.5 µg/ml Hydrocortisone, 10 ng/ml epidermal growth factor (EGF), 0.5 µg/ml Epinephrine, 5 µg/ml Transferrin and 4 ng/ml T3 (3,5,3'-triiodothyronine). Furthermore, 50 ng/ml Amphotericin B and 50 µg/ml Gentamycin were added according to the protocol delivered with the growth medium (Provitro GmbH, 2012, Zdrenka et al, 2012).

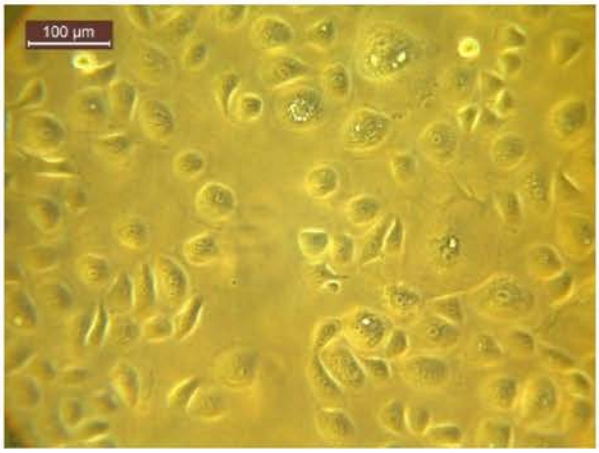


Fig. 13 HUEPC cells grown as a monolayer in a 75 cm² flask

3.1.3 HepG2 cells

HepG2 cells (Fig. 14) originate from a hepatocellular carcinoma of a 15 year old male Caucasian. They were obtained from ATCC (HB 8065; ATCC, Manassas, VA, USA) and cultured in Earle's minimal essential medium (MEM) enriched with 10% FBS, 0.5% Gentamycin, 1% L - glutamine, 1% non essential amino acids (NEAA) and 1% sodium pyruvate. The cells were incubated at 37 °C and 5% CO_2 atmosphere in 75 cm² flasks. They are adherent and generally grow in a monolayer, but as soon as HepG2 cells are confluent they build up multilayer (Zdrenka et al., 2012).

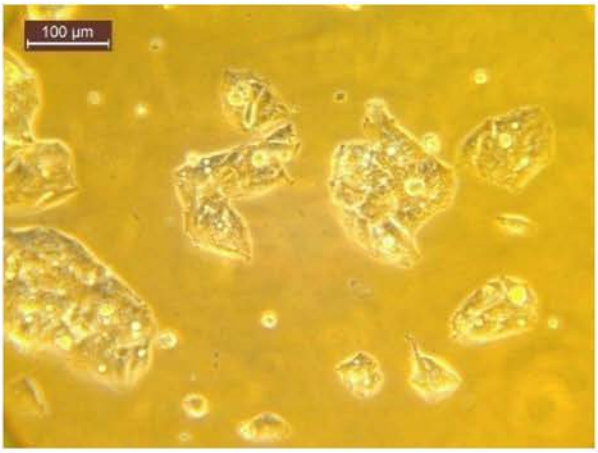


Fig. 14 HepG2 cells grown as a monolayer in a 75 cm² flask

3.2 Handling of the cell lines

3.2.1 Culture conditions and subculture

All cell lines were grown under typical cell culture conditions (37 °C, 5% CO_2, humidified incubator) and medium was replaced every 2 – 3 days. Cells were grown to 75 - 80% confluence. For subculture the cells were washed with phosphate buffered saline (PBS) and T24 and HepG2 cells were detached using 0.25% trypsine containing 0.1% 2-[2-[bis(carboxymethyl]amino]ethyl-(carboymethyl)amino]acetic acid (EDTA) and finally split into ¼ and transferred into new flasks (Zdrenka et al., 2012).

3.2.2 Cell exposure

Except for the chronic exposure experiments, the cells were seeded into cell culture devices according to the experimental purpose and incubated over night. Medium was then replaced by fresh culture medium containing the desired arsenic compound and concentration (exposure medium) and the cells were then incubated under cell culture conditions (Ch. 3.2.1).

For the chronic exposure experiments, 300,000 T24 cells were seeded into 75 cm^2 flasks and fed with 25 ml medium containing 50, 75, or 100 nM MMA(III), respectively. T24 cells fed with medium without any arsenic compound served as negative control. Once a week the cells were subcultured and fed with fresh exposure medium and 4 days later the exposure medium was replaced again. For cell detachment 0.25% trypsin without EDTA was used to prevent the complexation of arsenic (Zdrenka et al., 2012).

3.3 Applied methods

3.3.1 Determination of intracellular arsenic metabolism

Several studies report that humans, who are exposed to arsenic contaminated drinking water excrete many different arsenic metabolites such as MMA(V), DMA(V), and DMMTA(V). (Aposhian, 2000; Fillol, 2010). *In vivo* experiments showed that

DMA(V) caused bladder cancer in rats after exposure for a long time (Wei et al., 1999). Moreover, thiolated arsenicals (DMMTA(V)) could be detected in the urine of DMA(V) exposed rats (Yoshida et al., 1998). It still remains unknown, which arsenic species are responsible for the development of bladder cancer. Therefore, a new methodology for intracellular arsenic speciation *in vitro* was established and the arsenic metabolism in T24 cells was compared to that in HepG2 cells.

3.3.1.1 Cell exposure and sample preparation using Precellys®24 tissue homogeniser

Assay Principle:

Methylating HepG2 cells and non-methylating T24 cells were treated with 5 µM MMA(III) for various time periods. An exhaustive cell-washing process to remove all extracellular arsenic by dilution, complexation or covalent binding had to be explored to ensure that only arsenic originating form intracellular sources was detected. Therefore, the dilution ability was tested by repeated washing with PBS, and the complex formation of EDTA with the arsenic was investigated. None of those washing processes led to in satisfying results, as there were still considerable amounts of arsenic left. Finally, 2,3-bis(sulfanyl)propane-1-sulfonic acid (DMPS, Fig. 15), generally applied for the cure of heavy metal poisoning, was used for the removal of extracellular arsenic by covalently binding it to its sulfhydryl groups (Hippler et al., 2011).

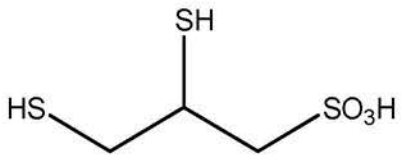


Fig. 15 Structure of DMPS (2,3-bis(sulfanyl)propane-1-sulfonic acid)

Furthermore, a suitable cell lysis method had to be explored. Therefore, freeze and thaw was tested, which did not lead to a complete cell lysis. In contrast, osmotic pressure resulted in a complete cell lysis, but the dilution effect was of a big disadvantage, because of the detection limits of the arsenic speciation and

quantification methodology. Finally, mechanical lysis using the Precellys®24 tissue homogeniser (Fig. 16) was examined (Hippler et al., 2011).

Fig. 16 Precellys®24 tissue homogeniser
The homogenizer (Pequlab Biotechnologie GmbH) (A) was used for mechanical lysis. The applied devices (C) contained ceramic beads (B) with a diameter of 1.4 mm. (Peqlab Biotechnologie GmbH, 2012)

The system is based on the destruction of cells and tissues by mechanical homogenisation using ceramic beads and shaking the samples with 6500 rpm. Subsequent centrifugation separated the non-soluble cell components from the soluble fraction (Hippler et al., 2011).

The non-soluble fraction consisted of membranes and other solid cellular structures. They were enzymatically digested using Proteinase K and the conjugated arsenic was released by oxidation with H_2O_2 (hydrogen peroxide) (Naranmandura et al., 2006; Hippler et al., 2011).

Methodology:

Cells were seeded into 150 cm² flasks and grown to confluence before experiments were performed. Both cell lines (T24 and HepG2) were incubated for 5 min up to 24 h in fresh growth medium containing 5 µM MMA(III) (exposure medium). The negative control consisted of cells incubated in fresh medium without any arsenic compound and subsequently they were handled the same way as the exposed cells. Additionally, a second negative control was trypsinised for cell counting.

After incubation the exposure medium was withdrawn and stored at -80°C. Cells were washed with 10 ml PBS and 10 ml Ampuwa (sterile, deionised water). For the next washing step 10 ml 0.1 mM DMPS were used to remove traces of extracellular uncombined arsenic ions. The last washing step with 10 ml PBS was carried out to remove the residues of DMPS before lysis (Fig. 17). All washing solutions were retained and stored at -80°C until arsenic speciation analysis (Hippler et al., 2011).

Fig. 17 Washing process prior to the intracellular arsenic speciation and quantification
Exhaustive cell washing ensures the removal of all extracellular arsenic residues after cell exposure (Hippler et al., 2011, modified)

Cell lysis was performed using the Precellys®24 tissue homogeniser as a tool for mechanical lysis. Therefore, the cells were first detached from the culture flasks using a cell scraper and transferred to 0.5 ml tubes containing ceramic beads with a diameter of 1.4 mm. Exhaustive homogenisation was obtained within three intervals of 20 s and 6500 rpm. Final centrifugation using a miniSpin plus centrifuge at 14.000 x g assured a complete separation of the cell lysates from membranes and other solid, insoluble cellular structures. This non-soluble fraction of each sample was then enzymatically digested using Proteinase K until the pellet was dissolved. Further oxidation with H_2O_2 (30%) assured the release of arsenic from peptides and other cellular molecules (Hippler et al., 2011).

3.3.1.2 Intracellular arsenic speciation and quantification using HPLC-ICP/MS

At this point I want to express my gratitude to my colleague and project partner J. Hippler from the Institute of Environmental Analytical Chemistry (University of Duisburg-Essen, Germany) under the head of Prof. Dr. Hirner, who kindly performed the experiments of the intracellular arsenic speciation and quantification and provided the data for the present study.

Assay Principle:

The High Performance Liquid Chromatography (HPLC) is based on the separation of a mixture of compounds by interaction of the analytes with the stationary phases. A pump moves the mobile phase and the analytes through the column. Depending on the type of column the adherence of the analytes differs, and in consequence there are analyte-specific retention times. By comparing with standard solutions or using data bases the analytes can be identified. Identification of the analytes can further be determined be coupling the HPLC with a detector such as an UV detector or a mass spectrometer (MS).

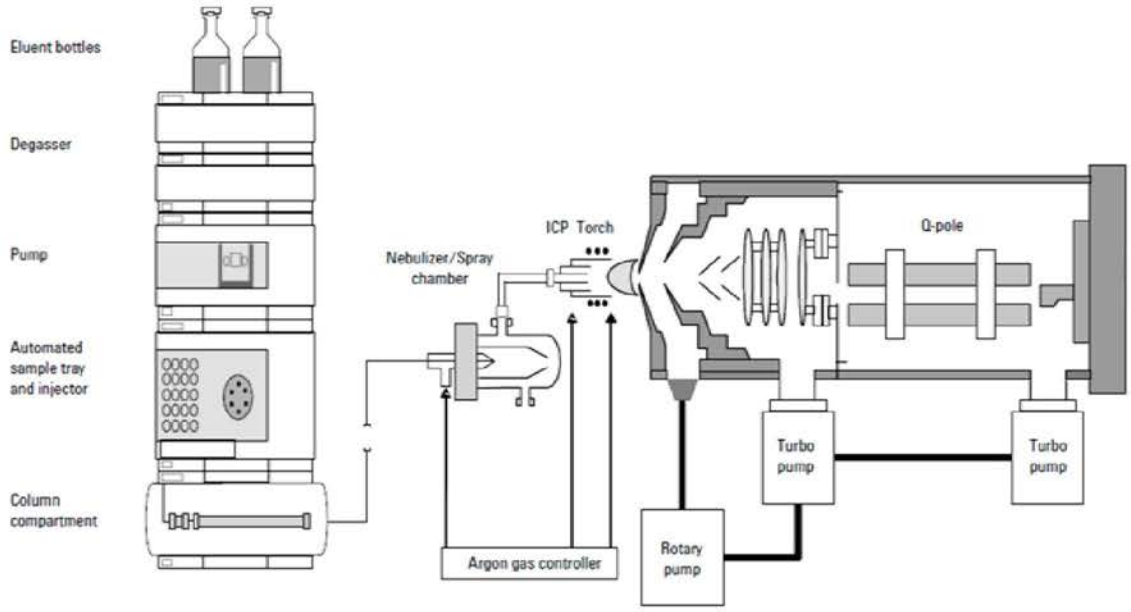


Fig. 18 HPLC-ICP/MS Coupling (Chen et al., 2005)

Using inductively coupled plasma mass spectrometry reveals the advantage of highly sensitive multi element analyses for a large number of samples and is often applied in the trace analysis of heavy metals such as mercury, lead, or cadmium. A plasma serves as an ionisation source and produces the ions, which are then recognised by the detector (m/z). Using ICP/MS most of the elements achieve detection limits in the magnitude of ng/l.

By coupling the ICP/MS with analytical separation methods such as HPLC, the system can be used for species analysis (Ammann, 2007).

The cell lysates and the digestion products of the non-soluble fraction were analysed by using HPLC-ICP/MS technique. The total and relative amount of the different

intracellular detected arsenic species and their development over time gives an interesting hint to the ability of intracellular biotransformation.

Methodology:

After exposure, lysis, and centrifugation all solutions and samples were stored at -80°C and thawed immediately before HPLC-ICP/MS analysis. The injected sample volume onto the HPLC-column was dependent on the expected arsenic content of the samples and counted 1 to 25 µl.

A multi-As species standard was used for quantification and contained 2 pg to 200 pg As(III), MMA(III), DMA(V), MMA(V), DMA(III), and As(V). Transient signals for As at m/z 75 in non-collision cell mode at dwell times of 100 ms were monitored and revealed the peak areas for arsenic quantification. External calibration resulted in an excellent linearity (e.g. MMA(V): $r^2 = 0.9999$; DMA(V): $r^2 = 0.999$). The limits of detection were calculated and revealed values of approximately 3 pg As, whereas the exact values varied slightly depending on the arsenic species. The conditions chosen for HPLC and ICP/MS are presented in (Tab. 3). To proof the reliability of the results, two replicate experiments were conducted (Hippler et al., 2011, as cited in Zdrenka et al., 2012).

Tab. 3 Conditions chosen for the HPLC and ICP/MS
(Hippler et al., 2011, modified, as cited in Zdrenka et al., 2012)

HPLC conditions		ICP/MS conditions	
HPLC Column	Phenomenex Luna 3µ C18(2) 100 Å	Forward power (RF)	1580 W
Column temperature	30 °C	Plasma gas rate (cool gas flow)	15 L Argon min^{-1}
Eluent flow rate	0.5 ml min^{-1}	Carrier gas flow rate	~ 0.8 L min^{-1}
Injection volume	1 – 25µl	Make-up gas flow rate	~ 0.25 L min^{-1}
		Sample depth	5.7 mm
Eluent:		Spray chamber	Quarz, cooled, 2 °C
Malonic acid	2 mM		
Tetrabutylammonium hydroxide (TBAH)	6 mM	Isotopes monitored	^{75}As, ^{35}Cl, $^{77}(^{40}Ar^{37}Cl)^{34}S$, ^{71}Ga, ^{73}Ge, ^{115}In, ^{103}Rh
Methanol	5 v/v%		
Water	95 v/v%		
pH	6.0		

3.3.2 Genotoxicity

3.3.2.1 Alkaline Comet Assay

Assay principle:

The Alkaline Comet Assay detects single and double strand breaks of the DNA by single cell electrophoresis. Hereby, the alkaline milieu rewinds the DNA and separates the strands. Because of the agarose density only small fragments of DNA can move in the electric field, whereas unbroken DNA stays densely bundled together (Fig. 19).

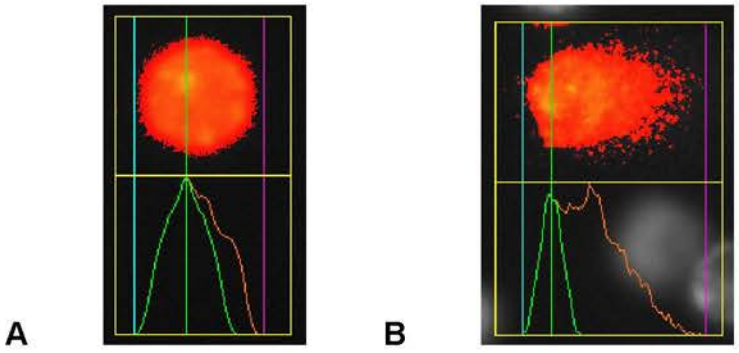


Fig. 19 Image analysis of the Alkaline Comet Assay
(A) Negative control: the DNA is densely bundled together, (B) positive control: the fragments of the damaged DNA moved in the electric field during electrophoresis

After staining the gels with a fluorescence dye intercalating into the DNA, image analysis can be performed. Hereby, several parameters such as tail length, tail fluorescence intensity etc. can be measured. Finally, the Olive Tail Moment can be calculated, which is defined as the product of the percentage of DNA in the tail multiplied by the tail length (Olive et al., 1991).

Methodology (T24 and HepG2 cells):

T24 and HepG2 cells were seeded with a density of 100,000 cells/2 ml medium into each well of a 24-well-plate and incubated over night. For exposure the cell culture medium was replaced by 2 ml of the exposure medium containing the arsenic compounds As(III), As(V), MMA(III), MMA(V), DMA(V) and TMAO in different concentrations. The negative control consisted of untreated cells, and positive control cells were exposed to 1 mg/ml ENU (N-ethyl-nitroso urea). For exposure to the

volatile DMA(III) 1,000,000 cells/10 ml medium were seeded into 25 cm² cell culture flasks with vent caps.

After incubation for 30 min and 60 min the exposure media were removed and the cells were trypsinised as described elsewhere (Ch. 3.2.1). The cells were resuspended in PBS and 5,000 cells were seeded into each agarose gel consisting of 0.79% low melting point agarose in PBS. The gels were solidified on ice and the cells were lysed overnight.

For electrophoresis the gels were washed with Ampuwa and incubated in 4°C cold electrophoresis solution at pH 13 for 15 min (T24) or 20 min (HepG2), respectively. After electrophoresis for 30 min (T24) or 20 min (HepG2), respectively, the gels were washed with Ampuwa and neutralised to pH 7.5. Subsequently, the gels were washed with Ampuwa again and incubated in 100% ethanol to remove the water residues. Finally, the gels were dried overnight at 4°C.

Methodology (HUEPC cells):

According to limited cell number HUEPC cells were seeded with a density of 15,000 cells/160 µl medium into each well of a 96-well-plate and incubated overnight. For exposure cell culture medium was replaced by 200 µl of the exposure medium containing the arsenic compounds As(III), As(V), MMA(III), MMA(V), DMA(V) and TMAO in different concentrations. The negative control consisted of untreated cells, and the positive control cells were exposed to 0.1 mg/ml ENU.

After incubation for 30 min exposure media were removed and the cells were detached as described above. The cells were resuspended in 20 µl PBS and seeded into each agarose gel consisting of 0.79% low melting point agarose in PBS. The gels were solidified on ice and the cells were lysed overnight.

For electrophoresis the gels were washed with Ampuwa and incubated in 4 °C cold electrophoresis solution at pH 13 for 15 min. After electrophoresis for 30 min the gels were washed with Ampuwa and neutralised to pH 7.5. Subsequently, the gels were washed with Ampuwa again and incubated in 100% ethanol to remove the water residues. Finally, the gels were now dried overnight at 4°C.

Image analysis was performed with Comet Assay IV Software (Perceptive Instruments Ltd., Haverhill, UK) using a fluorescence microscope and a digital

camera. Therefore, the cells were stained with SYBR GREEN and 50 cells/gel were evaluated. Statistical analysis was performed using Microsoft Excel and Graph Pad Prism. The experiments were performed in triplicate, but due to limited cell counts, HUEPC cells could be examined only once.

3.3.3 Intracellular COX-2 quantification

3.3.3.1 Cell lysis and sample preparation

Assay Principle:

The CytoBuster Protein Extraction Reagent is a mixture of detergents optimised for efficient extraction of soluble proteins from mammalian cells. The non-ionic composition of CytoBuster enables isolation of functionally active proteins. No further treatment such as sonication or freeze and thaw is needed (MerckKGaA, 2012).

Methodology:

T24 cells derived from the chronic treatment (Ch. 3.2.2) were seeded with 900,000 cells/150 cm² flask and grown to confluence for one week. In between, the cells were fed with fresh exposure media once. The exposure media always contained the same arsenic concentrations as during the chronic exposure before.

After one week of incubation the exposure media were removed and the cells were washed twice with 10 ml PBS. Subsequently, the cells were lysed with 1.5 ml CytoBuster. The detergent was incubated for 5-10 min at room temperature and the cells were detached using a cells craper. The total cell lysate was then brought into reaction tubes and the samples were centrifuged at 14,000 x g and room temperature for 30 min. Finally, the supernatant was aliquoted and stored at -80°C.

3.3.3.2 BCA (bicinchoninic acid) Assay

Assay Principle:

The BCA (bicinchoninic acid) Assay is generally determined for the quantitative analysis of proteins in complex samples such as cell lysates (Lottspeich & Engels, 2006). It is based on the Biuret Reaction and the selective complex formation of bicinchoninic acid and Copper(I)-Ions (Cu^+). The first step of the reaction is the

reduction of Cu^{2+} (originated from a $CuSO_4$-solution) by Cysteine-, Tyrosine- and Tryptohane residues, as well as by the peptide bonds. The resulting Cu^{+} forms a stable complex together with the BCA (Fig. 20), which can be quantitatively measured using a spectrophotometer at λ = 562 nm. This highly sensitive alternative to the Lowry-Assay was first published by Smith et al. (1985).

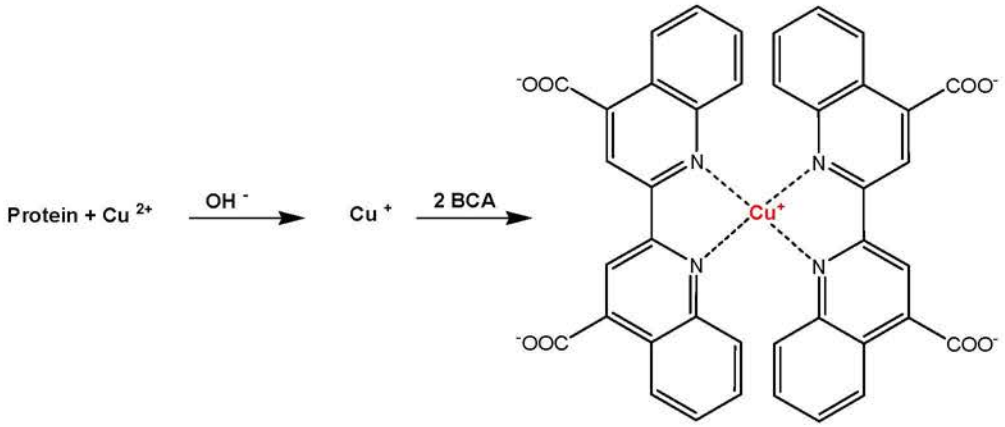


Fig. 20 The BCA (bicinchoninic acid) Assay
The BCA Assay is a combination of Biuret Reaction and the selective complex formation of Cu^{+} and BCA (Lottspeich & Engels, 2006).

Methodology:

For the quantitative analysis of the total protein content in the cell lysates the BCA Assay was applied. The chemicals were obtained from BioRad and the test was performed according to the provided protocol (BioRadLaboratories GmbH, 2012).

The samples had to be pre-diluted to a final concentration of 5%, and 5 µl each were added to a microtiter plate (Nunc Maxisorb). Additionally, a dilution series of BSA (bovine serum albumin) served as a standard for quantification. 25 µl of solution A' (1 ml solution A + 20 µl solution S; solution A: alkaline copper(II)-tartrat solution; solution S is necessary in the presence of detergents) were added, as well as 200 µl of solution B (Folin Reagent). After incubation for 15 min the absorbance was measured using a plate reader.

The analysis was performed in triplicates, and mean and standard deviation were evaluated. According to the standard curve the total protein concentration in the samples was calculated.

3.3.3.3 ELISA (enzyme-linked immunosorbent assay)

Assay Principle:

The *ELISA* is a largely known method for the quantitative analysis of biomolecules such as proteins. The principle is a 'sandwich-formation' of antibodies and the protein of interest (Fig. 21). Therefore, a microtiter plate is coated with an antibody that selectively binds the molecule of interest. After transferring the samples into the plate, a primary antibody is applied, that covers the bound protein. A secondary antibody conjugated with an enzyme binds now to the primary antibody and catalyses the colouric reaction of the applied substrate. The final addition of acid denaturises the enzyme and stops the reaction. The amount of the coloured reaction product can be quantitatively measured using a photometer (plate reader). The absorbance is directly proportional to the protein concentration in the analysed samples.

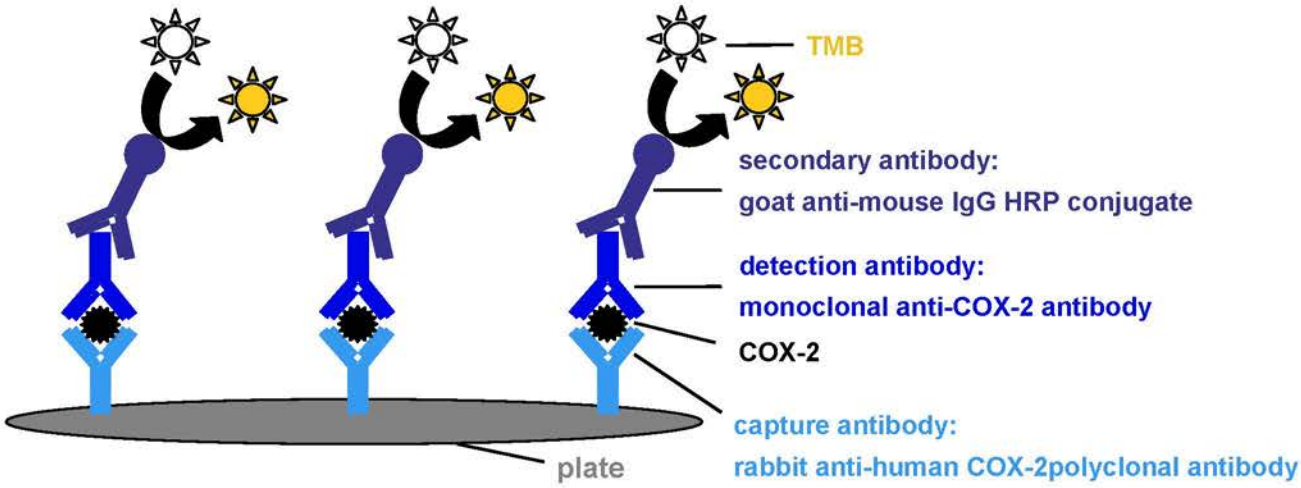


Fig. 21 Schematic illustration of the COX-2 ELISA

Methodology:

The COX-2 ELISA kit was obtained from Calbiochem (Merck KGaA, Darmstadt, Germany) and the experiment was performed according to the available protocol. 100 µl of the samples and the standards were transferred into the pre-coated microtiter plate and incubated for 90 min. After removing the supernatant the wells were washed three times with the wash buffer using an automated ELISA Washer. Subsequently, 100 µl of the primary antibody were added to each well. After incubation for 90 min the wells were washed again, and 100 µl of the secondary antibody with the HRP-conjugate (Horseradish peroxidase) were incubated for

another 60 min. After the final washing step 100 µl of the TMB substrate (3,3′,5,5′-tetramethylbenzidine) were added. The reaction was observed by measuring the absorbance of the standard curve at 360 nm. When the perfect absorbance was reached, the reaction was stopped by addition of 100 µl of the Stop-Solution (1 M H_2SO_4). Finally, the absorbance was measured at 450 nm.

The analysis was performed in duplicates and the mean and standard deviation were evaluated. According to the standard curve the COX-2 concentration in the samples was calculated. Finally, the COX-2 concentration was divided by the total protein concentration and the results received were given in µg COX-2/mg total protein.

3.3.4 miRNA analysis

At this point I want to express my gratitude to my colleagues and project partners Dr. D. G. Weber and Dr. O. Bryk from the Institute for Prevention and Occupational Medicine of the German Social Accident Insurance (Institute of the Ruhr-University Bochum, Germany) under the head of Dr. G. Johnen, who kindly performed the experiments of the miRNA analysis and provided the data for the present study.

3.3.4.1 Cell lysis and miRNA isolation

Assay Principle:

MiRNA isolation was performed by using the RNeasy Plus Mini Kit. Genomic DNA is easily removed without the need of DNase and highly purified miRNAs can be obtained from 1 Mio cells/sample. Fig. 22 schematically presents the isolation process (QIAGEN, 2012).

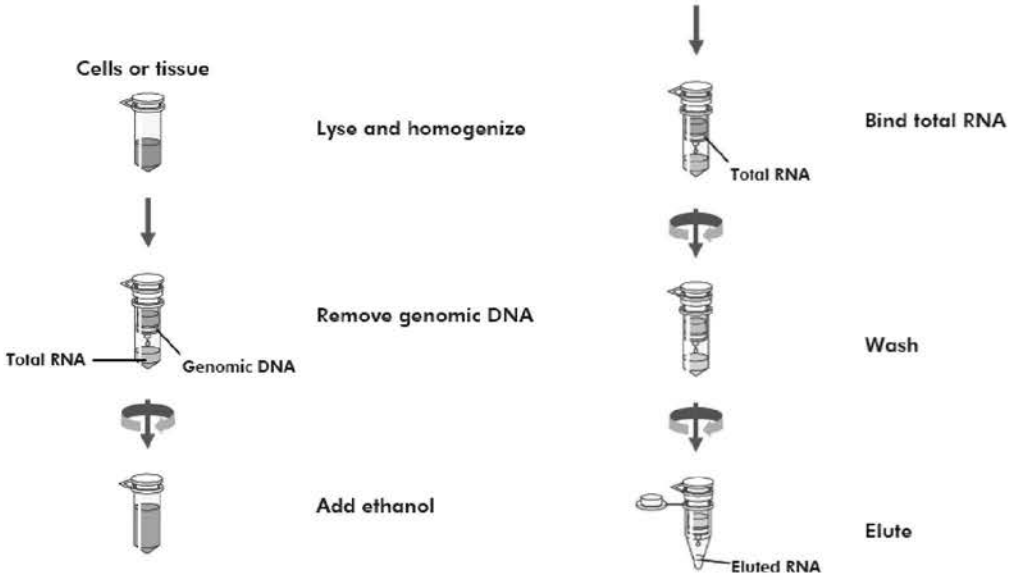


Fig. 22 Procedure of miRNA isolation using the RNeasy Plus Minikit (QIAGEN, 2012)

Methodology:

T24 cells obtained from the chronic treatment (Ch. 3.2.2) were harvested every 4 weeks. After trypsinisation, cells were resuspended in RNAlater and aliquoted into 1 Mio cell samples. The cells were stored at -20°C until analysis.

The miRNA isolation was performed by using the RNeasy Plus Mini Kit. The following procedure was conducted according to the user manual (QIAGEN, 2012), but with few minor modifications. The cells were centrifuged for 10 min at 400 x g and 4 °C. The supernatant was removed and the pellet was resuspended in 350 µl RLT+ buffer and 10 µl mercaptoethanol. The homogenised lysate was then transferred to a gDNA Eliminator spin column placed in a 2 ml collection tube and centrifuged for 1 min at 8,000 x g. After mixture of 350 µl ethanol (80%) with the flow-through a maximum volume of 700 µl was transferred to a RNeasy spin column placed in a 2 ml collection tube and again centrifuged for 1 min at 8,000 x g. Subsequently, the flow-through was mixed with a 1.4 times volume of ethanol absolute and transferred to an RNeasy Mini spin column placed in a 2 ml collection tube and centrifuged for 1 min at 8,000 x g once more. The desired miRNA was then fixed on the column and purified by washing with 500 µl RPE and 500 µl ethanol (80%). Finally, the desired miRNA was eluated with 50 µl RNase-free water.

3.3.4.2 Reverse Transcription

Assay Principle:

To amplify miRNAs they first have to be converted into DNA, because during RCR specific DNA dependent DNA polymerases are used, which are not able to amplify RNA. Hence, reverse transcription (Fig. 23) must be determined to produce a cDNA, which is complementary to the desired miRNA.

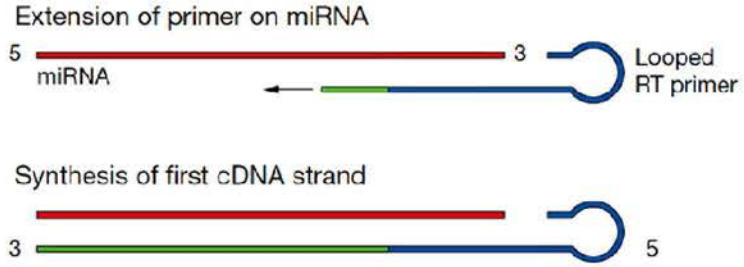


Fig. 23 Principle of the reverse transcription (Life Technologies Corporation, 2012)

Hereby, RNA dependent DNA polymerases are used, which are enzymes obtained from retroviruses. The resulting cDNA then serves as the parent material for the following PCR to finally amplify the sample.

Methodology:

Briefly, a mastermix of a stem-loop Reverse Transcription Primer, Reverse Transcription Buffer, dNTPs (2'-desoxynucleotide-5'-phosphates), MultiScribe Reverse Transcriptase, RNase inhibitor, and Nuclease-free water was mixed and aliquoted according to the sample quantity. 10 µl of the mastermix were added to 5 µl of the samples (template), and applied to the reverse transcription. Therefore, the thermocycler was run with one cycle only: 30 min at 16°C, 30 min at 42°C, and 5 min at 85°C. Finally, the samples were cooled down to 4°C.

3.3.4.3 Quantitative PCR (TaqMan assay)

Assay Principle:

The PCR (polymerase chain reaction) was originally developed in 1984 by scientists of the Cetus Corporation (Lexikon der Biochemie, 1999). It is determined to amplify a DNA fragment, which is located between two regions of a known nucleotidase-sequence within a complex DNA matrix. The source is not necessarily the isolated target gene, but also a complex DNA extract originated from a cell lysate can serve as raw material.

The first step of a PCR is always the denaturation of the target material to receive single strands. Therefore, the mixture is heated up to 92 – 96°C for 60 s, followed by a cool down to 55 – 60°C for 30 s. The primer now hybridise via hydrogen bonds to their complementary sequence of the target DNA. The primers are 20 – 25 nucleotide long, synthetically produced oligonucleotides, which are complementary to the flanked sequences of the target gene. They serve as the point of initialisation for the polymerase and are elongated towards the 3' → 5' direction during the third and last step of the PCR. Generally, the Taq-DNA-Polymerase isolated from the bacterium *Thermus aquaticus* is applied (Saiki et al.,1988), as it survives the heat during the denaturation step. This is prerequisite for the automatisation of the PCR. The Taq-Polymerase connects the complementary dNTPs to the 3'-hydroxy-ends of the new strand. For this reaction a temperature of 72°C for 1 – 3 min is required, depending on the length of the amplificated matrix. The whole process is repeated a numerous times, whereas every repetition doubles the material.

TaqMan Assay

The TaqMan Assay is a real time PCR for the quantitative analysis of the target DNA matrix with its detection limits in the order of magnitude of femto- and attograms (Lottspeich & Engels, 2006). The amplification products are already measured during the PCR (homogenous quantitative analysis) without the removal of the reaction educts and the addition of detection reagents. This closed system prevents a sample contamination.

For the detection a probe is applied, which adheres to the target sequence during the amplification. Such a probe is generally a combination of a fluorescence dye (reporter) and a quencher, whereas the latter absorbs the emitted fluorescence light

from the dye. Following hybridisation to the target molecule, the probe is splitted by the 5'-3'-exonuclease activity of the Taq-Polymerase and a fluorescent nucleotide is released (Fig. 24).

The fluorescence can be quantitatively detected and directly correlated with the amount of amplificated hybrid complexes. The quantity is given as the cT-value, which indicates the amount of necessary PCR cycles to exceed a threshold. Plotting the cT-values half-logarithmic against the amount of copies in the raw material, a linear connection is resulted. Using a calibration with external standards or the co-amplification with internal standards, the number of copies of the analysed samples can be determined.

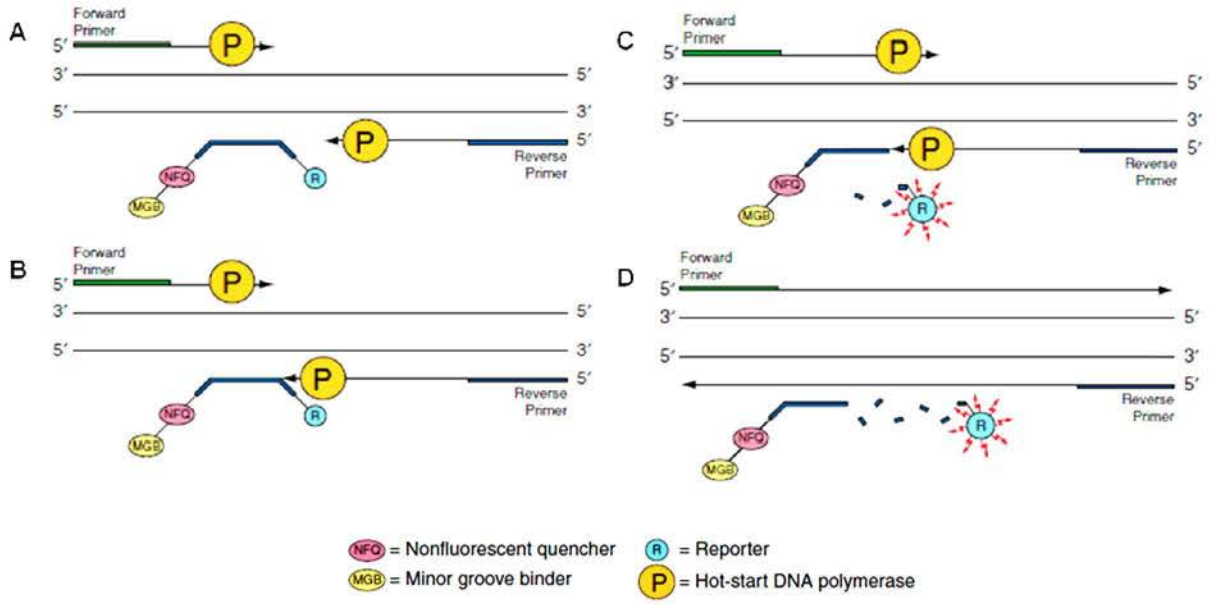


Fig. 24 Principle of the TaqMan Assay (Life Technologies Corporation, 2012)

Methodology:

Briefly, a mastermix containing TaqMan Assay Solution, TaqMan Buffer, and Nuclease-free water was prepared and transferred to optical 8-Tube-stripes (15 µl). 5 µl of the samples (template) were added to each tube, whereas two replicates of each sample were prepared. Finally, the samples were loaded into the real-time PCR device and the PCR was started with 10 min at 95°C. Subsequently, 40 cycles were run with 15 s at 95°C and 1 min at 60°C.

For the quantification of miRNA changes in the T24 cells, the results were normalised according to the ΔΔCt-Method of Livak & Schmittgen (2001) (Equation 3):

(3) $\Delta\Delta Ct = (Ct_{miRNA} - Ct_{RNU})_{sample} - (Ct_{miRNA} - Ct_{RNU})_{control}$

whereas CtRNU is the mean value of the small nuclear RNAs RNU44 and RNU48, which were selected for the normalisation as they exhibited stable expression patterns throughout the exposure to different concentrations of MMA(III).

3.3.5 DNA methylation

At this point I want to express my gratitude to my colleagues and project partners P. Rozynek and Y. von der Gathen from the Institute for Prevention and Occupational Medicine of the German Social Accident Insurance (Institute of the Ruhr-University Bochum, Germany) under the head of Dr. G. Johnen, who kindly performed the experiments of DNA methylation analysis and provided the data for the present study.

3.3.5.1 Cell lysis and DNA isolation

Assay Principle:

Cell lysis is a mandatory step in DNA isolation procedures and can be performed with detergents such as the ATL buffer. In addition, cellular proteins have to be broken down to avoid DNA degradation caused by DNAses. For this purpose the Proteinase K, a serin protease isolated from the fungus *Engyodontium album* (*formerly Tritirachium album*) (Ebeling et al., 1974), is generally added to the samples. After incubation and degradation of the samples, DNA isolation can easily be performed by using the QIAcube. This device enables fully automated sample preparation of up to 12 samples per run. In this studythe QIAamp DNA Mini QIAcube Kit was applied. It simplifies DNA isolation from human tissue samples with fast spin-column procedures. No phenol-chloroform extraction is required, as the DNA binds specifically to the QIAamp silica-gel membrane while contaminants pass through. PCR inhibitors, such as divalent cations and proteins, are completely removed in two

wash steps. Pure DNA is eluted and genomic DNA sized up to 50 kb ready to be used in PCR (QIAGEN, 2012) is yielded.

Fig.25 QIAcube for automated DNA isolation procedures (QIAGEN, 2012)

Methodology:

T24 cells obtained from the chronic treatment were harvested every 4 weeks (Ch. 3.2.2). After trypsinisation, cells were resuspended in PBS and stored at -20 °C until analysis.

Samples of 2 Mio cells were centrifuged for 1 min at 11,000 rpm and incubated with 180 µl Buffer ATL and Proteinase K at 56 °C. As soon as the samles were no longer viscid, DNA isolation was performed using the QiaCube in combination with the QIAamp DNA Mini QIAcube Kit. The settings used were those, suggested by the supplier (QIAGEN, 2012). The final elution volume was set at 200 µl. Using the NanoDrop the DNA concentration was determined at OD 260 nm.

3.3.5.2 Bisulfite sequencing

Assay Principle:

The methodology of bisulfite sequencing allows the detection of DNA methylation in a given substrate. The treatment with bisulfite (sodiumhydrogensulfite) converts unmethylated cytosines into uracil (Fig. 26), whereas methylated cytosines are protected from this reaction. After amplification by PCR and sequencing, the methylated cytosines will be sequenced as cytosines and unmethylated cytosines appear as thymines.

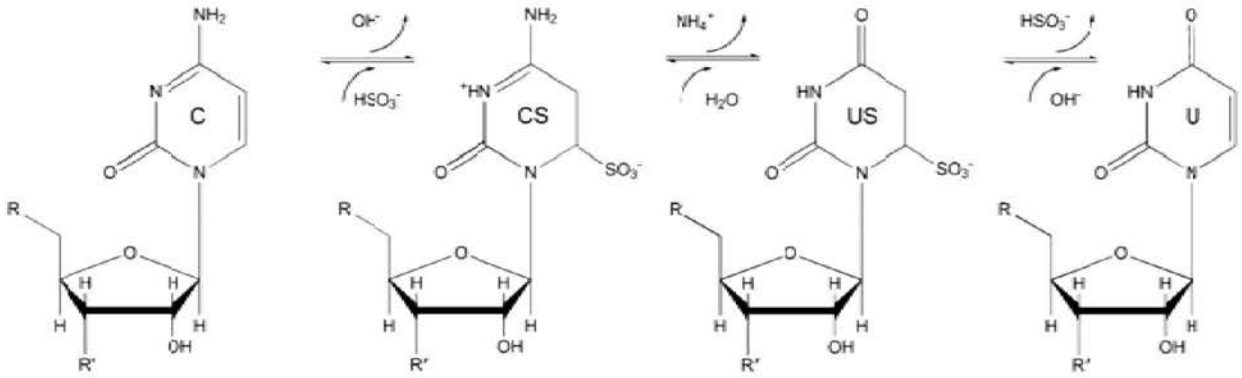


Fig. 26 Bisulfite conversion reaction (Schaefer et al., 2009)

The QIAGEN EpiTect Bisulfite Kit exhibits the advantage of a fast procedure as automated conversion and purification of bisulfite-converted DNA is possible on the QIAcube (Fig. 27). After bisulfite modification the converted single-stranded DNA binds to the membrane of an EpiTect spin column. The membrane-bound DNA is washed and desulfonated, and purified converted DNA is eluted from the spin column (QIAGEN, 2012).

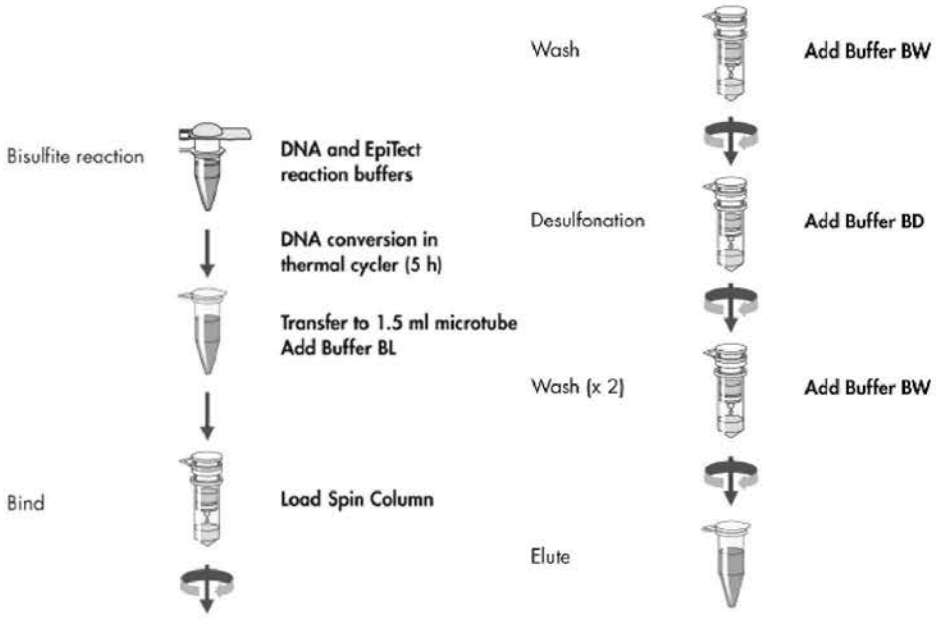


Fig. 27 Procedure of bisulfite conversion using the QIAGEN Epitect Bisulfite Kit (QIAGEN, 2012)

The principle of pyrosequencing can be divided into 5 steps (Fig. 28). In a first step a sequencing primer is hybridised to a single-stranded PCR amplicon that serves as a template, followed by incubation with the enzymes, DNA polymerase, adenosine

triphosphate (ATP) sulfurylase, luciferase, and apyrase as well as the substrates, adenosine 5' phosphosulfate (APS), and luciferin. Subsequently, the first dNTP is added to the reaction. If the dNTP is complementary to the base in the template strand, the DNA polymerase catalyses its incorporation. Each incorporation event is accompanied by the release of pyrophosphates in equimolar quantities to the amount of incorporated nucleotides.

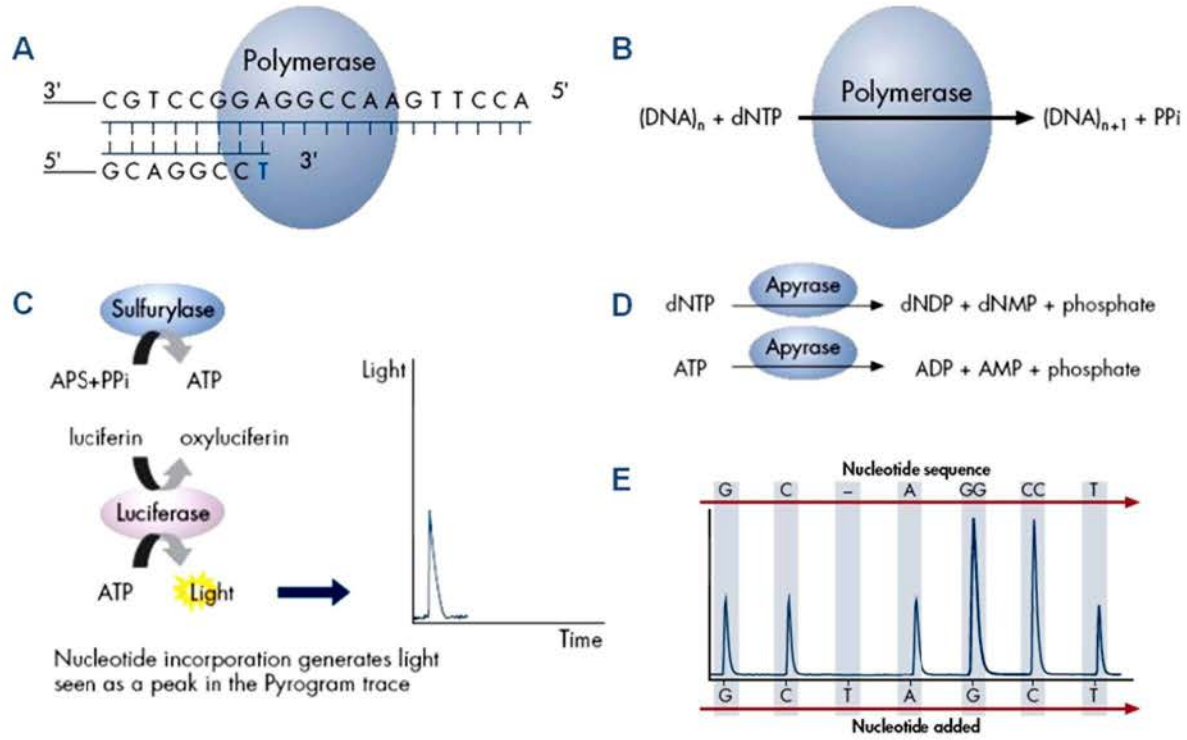


Fig. 28 The 5 steps of the pyrosequencing reaction (QIAGEN, 2012)

ATP sulfurylase converts the pyrophosphates to ATP in the presence of adenosine APS. This ATP drives the luciferase-mediated conversion of luciferin to oxyluciferin that generates visible light in amounts proportional to the amount of ATP. The light produced in the luciferase-catalysed reaction is detected by a chip and seen as a peak in the pyrogram. The height of each peak is proportional to the number of nucleotides incorporated. The apyrase continuously degrades unincorporated nucleotides and ATP. When degradation is complete, another nucleotide is added. Finally, the addition of dNTPs is performed sequentially. Hereby, desoxyadenosine alfa-thio triphosphate (dATP·S) is used as a substitute for the natural

desoxyadenosine triphosphate (dATP), since it is efficiently used by the DNA polymerase, but not recognized by the luciferase. As the process continues, the complementary DNA strand is built up and the nucleotide sequence is determined from the signal peaks in the pyrogram (QIAGEN, 2012).

Methodology:

For the bisulfite modification the QIAGEN Epitect Bisulfite Kit was used exactly according to the suppliers protocol (QIAGEN, 2012). Following bisulfite modification and purification of the converted DNA, an assay-specific PCR was performed by using the AmpliTaq Gold DNA Polymerase and a reverse-primer, which is 5'-biotinylated. Denaturation was achieved at 95°C for 20 s, followed by the annealing at 50 - 60°C for 20 s. Elongation of the strands was accomplished at 72°C for 20 s. 50 PCR cycles were needed to ensure that most of the primers (especially the biotinylated ones) are consumed to minimise the background noise for the following pyrosequencing step. After the last cycle, the samples were first kept at 72°C for 5 min before they were cooled down to 8°C.

After success monitoring via agarose gel electrophoresis, the amplicons were denaturated and single strands were obtained by using the Biotage Sample-Prep Workstation according to the supplier's protocol (QIAGEN, 2012). Pyrosequencing was determined using the Biotage Pyrosequencer PSQ 96MA and the PyroMark Gold reagents. The assay is designed for the detection of 5 - 15 CpGs per promoter region and analyses the methylation of every single CpG.

3.3.6 In vitro mammalian cell transformation test

Assay Principle:

The ability of contact inhibition is one of the manifold mechanisms to prevent inappropriate cell proliferation and can easily be observed in two-dimensional cell cultures, which stop further proliferation as soon as a confluent cell monolayer is built up (Hanahan & Weinberg, 2011). In neoplasias, cells lose the ability of contact inhibition. There are simple *in vitro* experiments to assay this phenomenon. Cells

grown to confluence in general stop proliferation, but after transformation and a loss of contact inhibition they continue proliferation and form colonies.

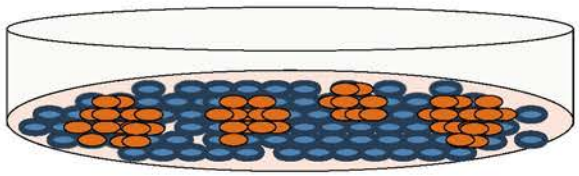


Fig. 29 Cell culture experiment to assay the loss of contact inhibition in vitro (blue: confluent cell monolayer, red: colonies built up after loss of contact inhibition).

This method is, among others, mentioned in the EU Method B.21 and can be found in Dir 88/303/EEC (OJ L 133 1988). However, the guideline emphasises that the available methods for the detection of phenotypical changes alone have not been established to create a mechanistic link with cancer, but nevertheless, they might be capable of detecting tumour promoters.

Methodology:

T24 cells obtained from the chronic exposure (Ch. 3.2.2) were grown to confluence in 12.5 cm² flasks and fed twice a week with 12.5 ml fresh medium containing 50, 75, or 100 nM MMA(III), respectively. UROtsa cells fed with medium without any arsenic compound served as negative control. Image analysis was performed subsequently after 2 weeks of incubation using an inverted microscope combined with a Leica digital camera.

3.3.7 Colony formation assay

Assay Principle:

When a normal cell is transformed into a cancer cell it loses its restraint to grow adherent in monolayers. After cell transformation, which is a step towards malignancy, the cells gain the ability to grow anchorage-independent in soft agar and form colonies. With help of the colony formation assay (Bredfeldt et al., 2006) the development of an anchorage-independent growth after chronic arsenic exposure can be determined (Zdrenka et al., 2012). Therefore, T24 cells obtained from the chronic exposure experiment were seeded into soft agar and cultured for two weeks.

With the loss of the anchorage dependent growth the cells are able to proliferate and build cell colonies, otherwise the cells underlie apoptosis. To avoid false positive effects because of the high passages two controls were included into the assay: the untreated negative control cultured under the same conditions like the treated cells, as well as untreated cells of a young passage.
This method is, among others, mentioned in the EU Method B.21 and can be found in Dir 88/303/EEC (OJ L 133 1988). However, the guideline emphasises that the available methods for the detection of phenotypical changes alone have not been established to create a mechanistic link with cancer, but nevertheless, they might be capable of detecting tumour promoters.

Methodology:

The colony formation assay was prepared in a 24-well plate. First, 500 µL of a base agar containing 0.6% low melting point (LMP) agarose in cell culture medium were added to each well, solidified at room temperature, and sterilised under UV light for at least 4 hours.

T24 cells derived from the chronic exposure experiment (Ch. 3.2.2) were seeded with a density of 10,000 cells/500 µL top agar (0.3% LMP agarose in cell culture medium) into each well. Additional wells were prepared without cells containing only base and top agar for background detection. The cells were fed with 250 µL of fresh cell culture medium every 3 – 4 days, and image analysis was performed subsequently after 2 weeks of incubation using an inverted microscope combined with a Leica digital camera (Zdrenka et al., 2012).

3.3.8 Cellular migration and invasion

Assay Principle:

The ability of invasion is a particular characteristic of malignant transformed cells. The cells are now able to detach from the parent tissue and exhibit protease activity to move actively through a biological layer. After migration through the body circulation they exhibit the skill, to attach and integrate into a new tissue. The ability of invasion provides the basis for the metastasing properties of tumours.

For the chronic low-dose exposure it was interesting to determine, whether the chronic treatment with MMA(III) can cause such changes. Therefore, the xCELLigence DP system (Roche Applied Science, Mannheim, Germany) (Fig. 30) with its CIM-Plates (Fig. 31) was used.

Fig. 30 xCELLigence DP System with control unit and analyser
(Roche Diagnostics GmbH, 2009)

These devices consist of a two-chamber-system separated with a microporous membrane. The membrane is combined with a gold electrode on the lower side. The cells are seeded into the upper chamber, whereas the lower chamber is filled with cell culture medium only. For simulating a biological layer both sides of the membrane were coated with collagen, since collagen IV is the major component of the basement membrane (Du et al., 2009).

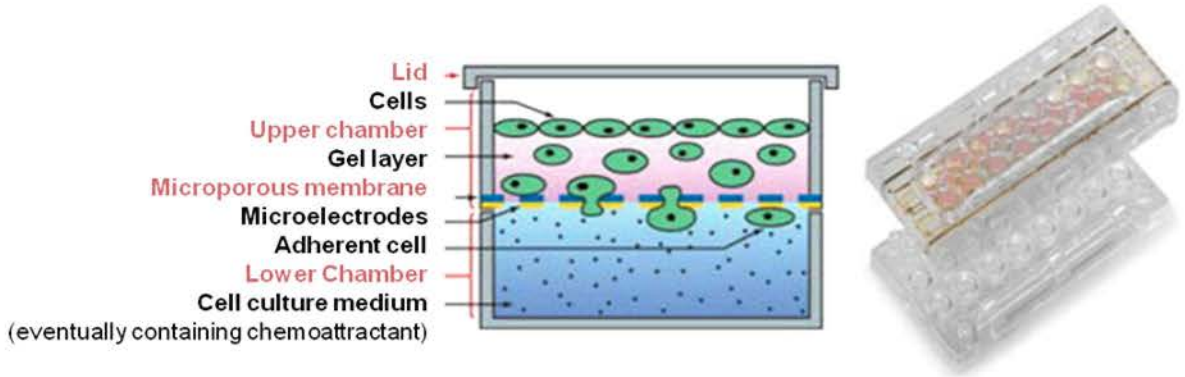


Fig. 31 xCELLigence CIM-Plate system for invasion analysis
(Bird & Kirstein, 2009, modified)

The cells, which gained the ability of invasion, can now move through the collagen layer and pass the microporous membrane, but only the cells which then adhere at

the lower side of the membrane at the electrode are detected. Particles, cell fragments, and cells, which cannot attach, will not be detected.

Attached cells are detected because they cause a relative change in the measured electrical impedance, which is displayed via the dimensionless parameter termed Cell Index (CI). In the absence of cells or when the cells are not well adhered on the electrodes, the CI is zero. In contrast, the attachment of cells under the same physiological conditions results in an CI above zero, whereas the CI is directly proportional to the cell count and cell adhesion (Fig. 32; Abassi, 2008).

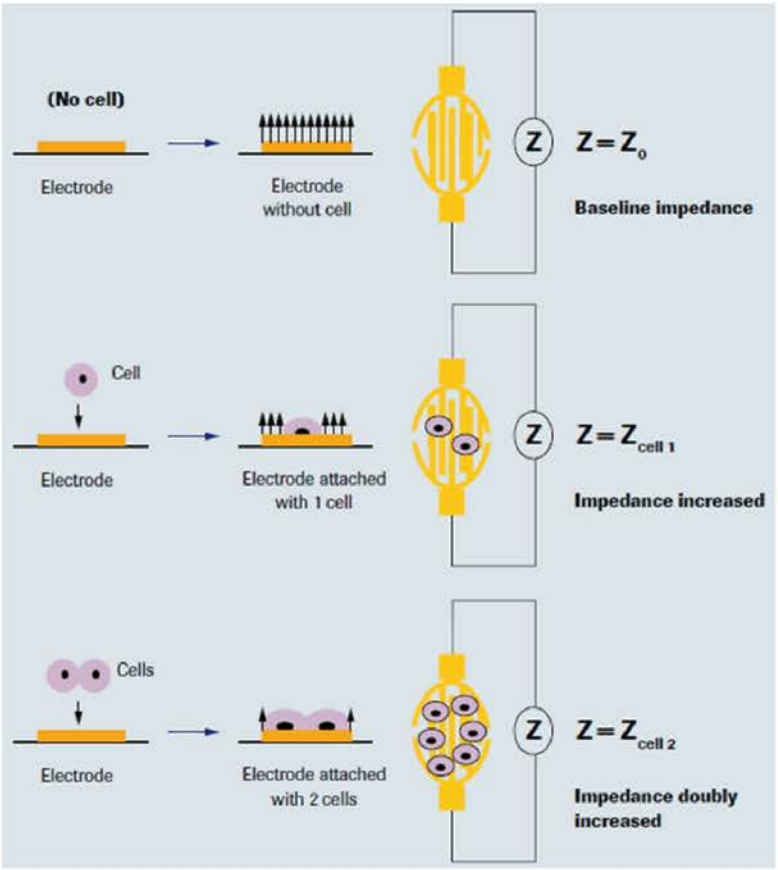


Fig. 32 Principle of the measurement of the Cell Index using the xCELLigence system (Abassi, 2008)

The measurement was conducted by using the provided software. The advantage of this system is not only the guarantee, that only actively invading cells are detected, but also that this method is not an endpoint assay. During the whole experiment the system measures label-free in arbitrary intervals, so that a time dependent development can be observed.

Methodology:

For the detection of invasion the xCELLigence DP system was conducted. Therefore, CIM-Plates were coated with 50 µl of 0.02% collagen on each side of the microporous membrane for 1 h. After removing the collagen residues the CIM-Plates were dried for approximately 1 h at room temperature under the laminar flow. In between T24 cells originated from the chronic exposure were trypsinised as described elsewhere (Ch. 3.2.2) and brought to a concentration of 1.5 Mio cells/ml. For the detection of migration uncoated CIM-Plates were conducted.

160 µl of the pre-warmed cell culture medium were added to each well of the lower chamber and 100 µl were added to each well of the upper chamber. The CIM-Plates were then placed into the xCELLigence device and the background was measured. Now 100 µl of the prepared cell suspensions were added (duplicate wells), and the CIM-Plates were placed into the xCELLigence device again. The measurement was performed for 24 h with intervals of 15 min (Zdrenka et a., 2012).

3.4 Statistics

All experiments were performed in triplicate, unless stated otherwise. The statistical evaluation for the Alkaline Comet Assay was performed using GraphPad Prism (GraphPad Software, Inc., CA, USA). The mean values of the detected Olive Tail Moments are presented in bar graphs with the standard error of mean (SEM). For statistical analysis the non-parametric Mann-Whitney-Test was applied, which approximates the Gaussian distribution for more than 20 random samples and compares each test group to the untreated control group. Probability values (p) are given as follows:

$p > 0.05$ non-significant, $p \leq 0.05$ *, $p \leq 0.01$ **, $p \leq 0.001$ ***

The quantification of miRNAs was determined using the ΔΔCt-Method. With $2^{-\Delta\Delta Ct}$ the relative quantities (RQ) of samples of the treated cells were compared to those of the untreated control cells. Significance levels according to Mattie et al. (2006) were set at $RQ < 0{,}5$ (decrease) and $RQ > 2{,}0$ (increase).

4 Results

In this study several endpoints *in vitro* were analysed to gain further knowledge about the processes ongoing during arsenic-induced bladder carcinogenesis. First, the intracellular metabolism of MMA(III), one of the most important metabolites during arsenic intoxication, was determined in human urothelial cells (T24) and compared to that of hepatocytes (HepG2). Furthermore, acute genotoxic effects of the arsenic species As(III), MMA(III), DMA(III), As(V), MMA(V), DMA(V), and TMAO were analysed using the Alkaline Comet Assay. A dose-response relationship was examined, as well as the impact of the exposure duration. Additionally, genotoxicity in T24 cells was compared to that in HepG2 cells, which are known to exhibit metabolic properties, and primary human uroepithelial cells (HUEPC).

Moreover, possible effects resulting from chronic low-dose exposure to MMA(III) were analysed. Those included epigenetic events such as the regulation status of COX-2 protein, miRNA analysis, DNA methylation patterns (both global and specific promoter methylation), as well as phenotypic alterations such as the loss of contact inhibition, anchorage-independent growth, and migrating and invading properties.

The study addresses some of the most important effects during carcinogenesis and reveals a strong overview over molecular effects following arsenic exposure.

4.1 Intracellular arsenic speciation and quantification

At this point I want to express my gratitude to my colleague and project partner J. Hippler from the Institute of Environmental Analytical Chemistry (University of Duisburg-Essen, Germany) under the head of Prof. Dr. Hirner, who kindly performed the experiments of the intracellular arsenic speciation and quantification using HPLC-ICP/MS and provided the data for the present study.

First data were already published in Hippler et al. (2011), and further results were presented and discussed in Zdrenka et al. (2012), wherefrom the respective information was recited in the following chapter.

Results of the intracellular arsenic speciation and quantification

Using HPLC-ICP/MS analysis enabled the detection of more than 99.99% of the total arsenic in the non-soluble fraction of both cell lines, and only 0.003% and 0.01% of the total arsenic in the soluble fractions of T24 cells and HepG2 cells, respectively. While in the non-soluble fraction of T24 cells the arsenic content consisted only of a monomethylated species, in HepG2 cells a time dependent occurrence of a dimethylated arsenic species additionally to monomethylated arsenic was observed (Fig. 33). The differentiation between trivalent and pentavalent arsenic metabolites was impossible due to their oxidative release from the cellular structures (Zdrenka et al., 2012).

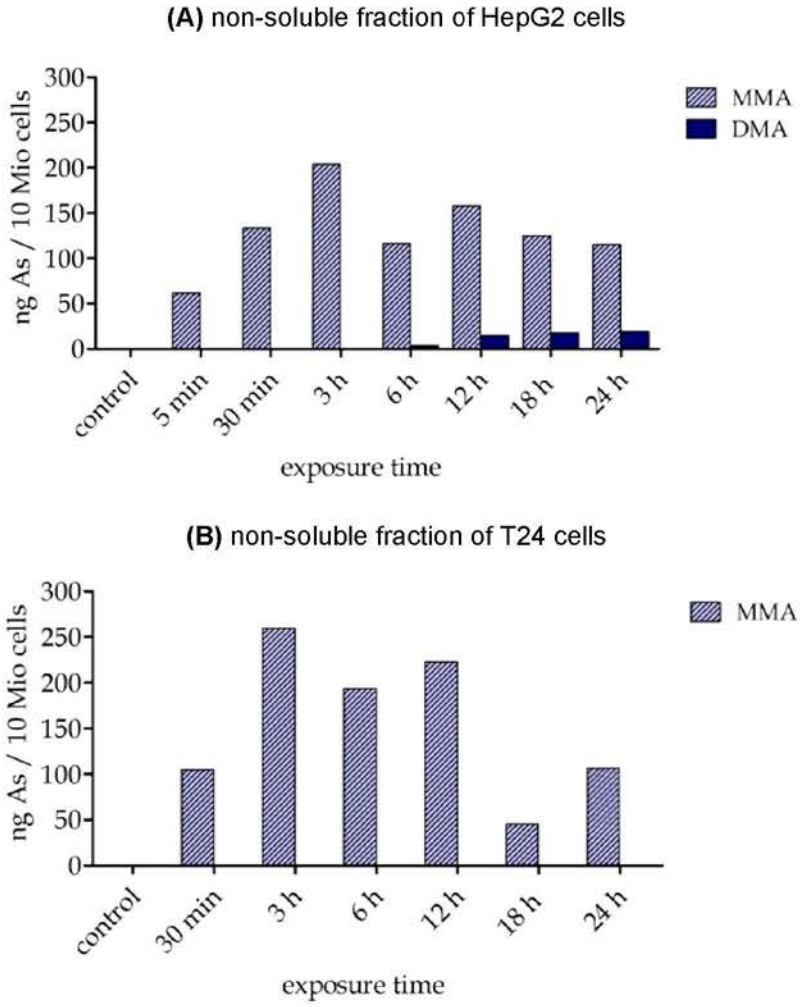


Fig. 33 Quantification of the metabolites in the non-soluble fraction after exposure of HepG2 (A) and T24 (B) cells to 5 µM MMA(III)

The analysis was performed using HPLC-ICP/MS technique (Zdrenka et al., 2012). Since this experiment was conducted once only, no statistical analysis was performed

In the soluble fractions of both HepG2 and T24 cells only pentavalent arsenic species were detected (Fig. 34). In HepG2 cells a time dependent increase and decrease of MMA(V) was observed. Additionally, the increase of DMA(V) by time was discovered. In contrast, in T24 cells only MMA(V) but no DMA(V) was detected. The occurrence of MMA(III) after 18 and 24 hours of exposure is believed to be the result of cytotoxic effects and due to membrane damage (Zdrenka et al., 2012).

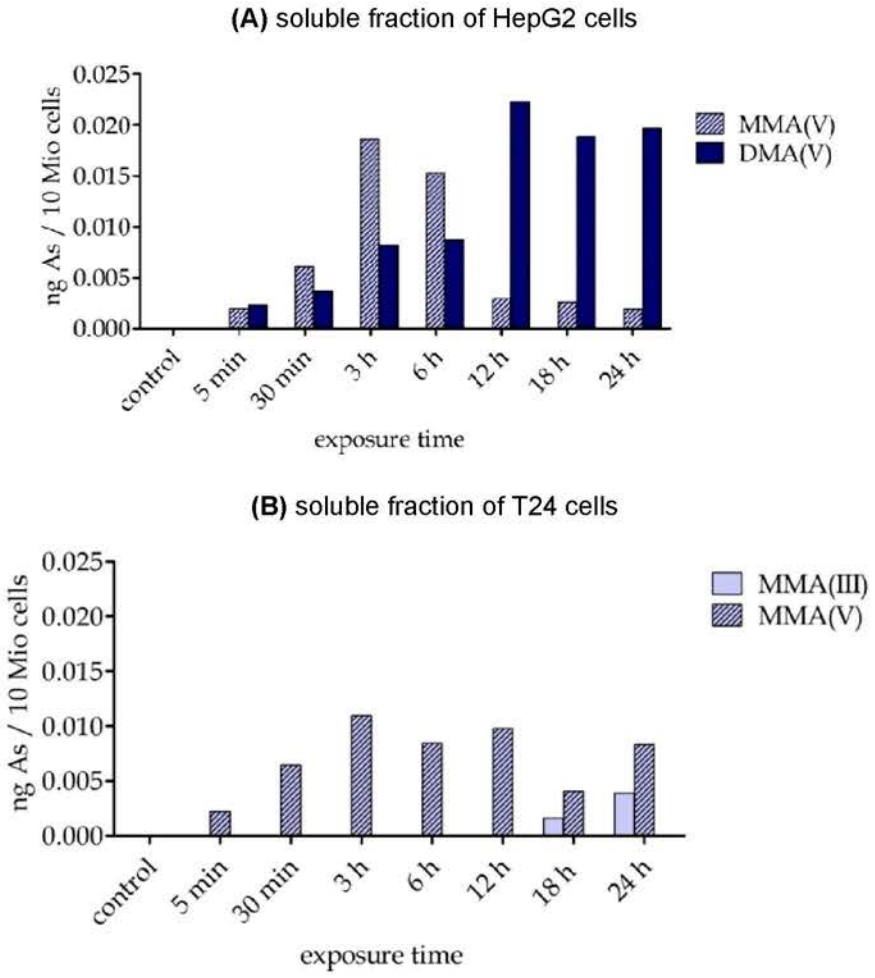


Fig. 34 Quantification of the metabolites in the soluble fraction after exposure of HepG2 (A) and T24 cells (B) to 5 µM MMA(III)

The analysis was performed using HPLC-ICP/MS technique (Zdrenka et al., 2012). Since this experiment was conducted once only, no statistical analysis was performed.

4.2 *Alkaline Comet Assay*

First data were already presented and discussed in Zdrenka et al. (2012), wherefrom the respective information was recited in the following chapter.

The Alkaline Comet Assay was conducted to examine the genotoxic effects of different arsenic metabolites. Testing the trivalent species revealed significant single and double strand breaks in T24 cells already after 30 min of exposure (Fig. 35).

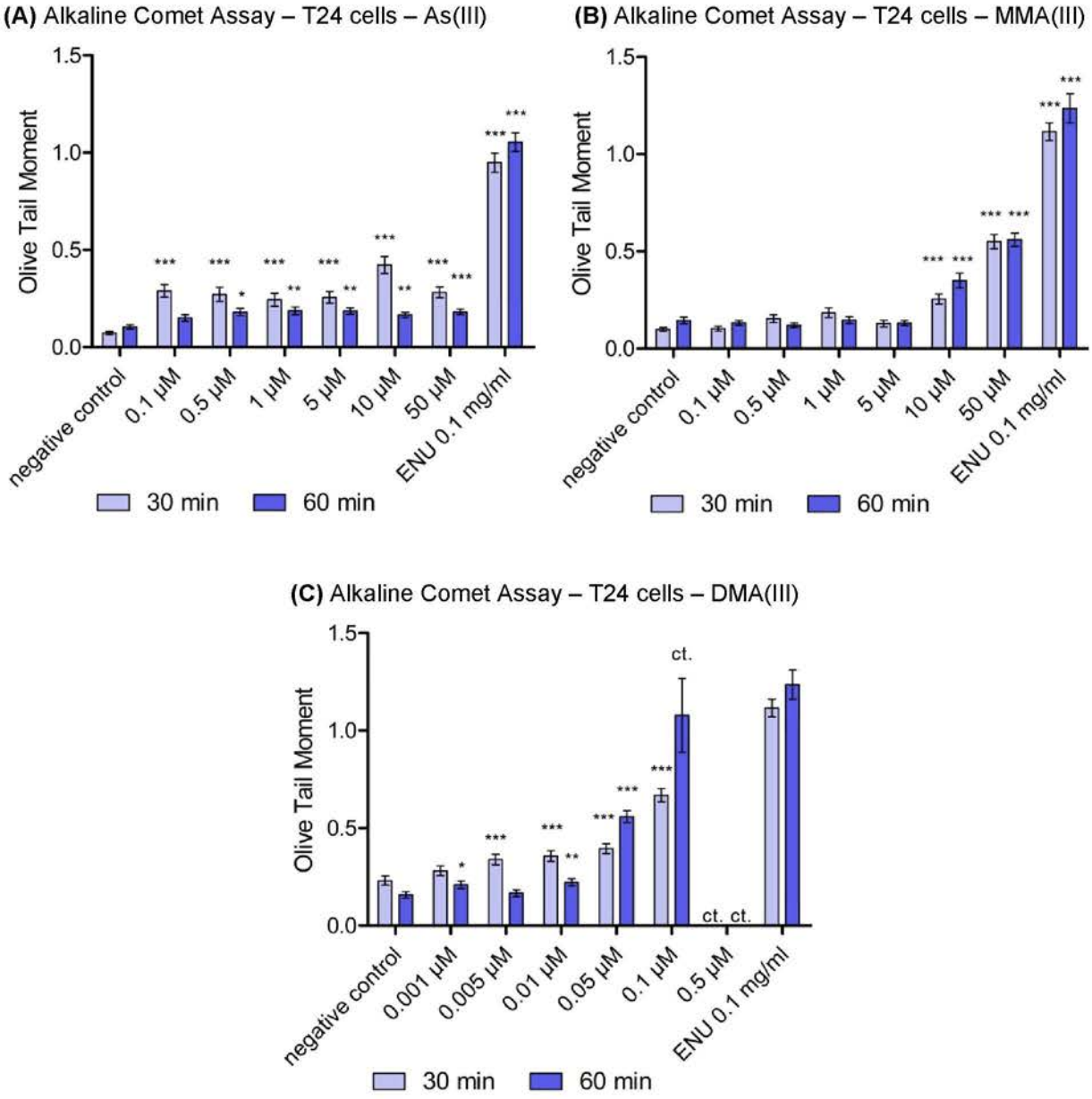


Fig. 35 DNA damage in T24 cells after 30 and 60 min of treatment with trivalent arsenic species
The Alkaline Comet Assay was conducted to assay single and double strand breaks of the DNA after 30 exposure As(III) (A), MMA(III) (B), and DMA(III) (C). The DNA damage is given in the Olive Tail Momen
(mean ± SEM with p > 0,05: non-significant, p ≤ 0,05: *, p ≤ 0,01: **, p ≤ 0,001: *, n = 150 cells).**

It seems that genotoxicity decreases with longer exposure time. This effect is misleading due to the occurrence of first cytotoxic effects already at the concentrations chosen. Microscopic evaluation revealed cells with decreased size and altered appearance, their number increasing proportional to the exposure concentration. It is assumed that the cells, which suffered severe damage, adhered only very loosely, and hence, these cells were lost during sample preparation, e.g. during removal of the exposure medium, cell washing, or trypsination. However, testing up to cytotoxic concentrations is in compliance with methods recommended for regulatory purposes (European Union [EU], 2008).

Although biotransformation was initially discussed to serve as a detoxification process, MMA(III) still is highly genotoxic and DMA(III) even exhibits the most genotoxic effects. In contrast, pentavalent arsenic species did not show any genotoxic effect except for As(V) at very high concentrations (Fig. 36) (Zdrenka et al., 2012).

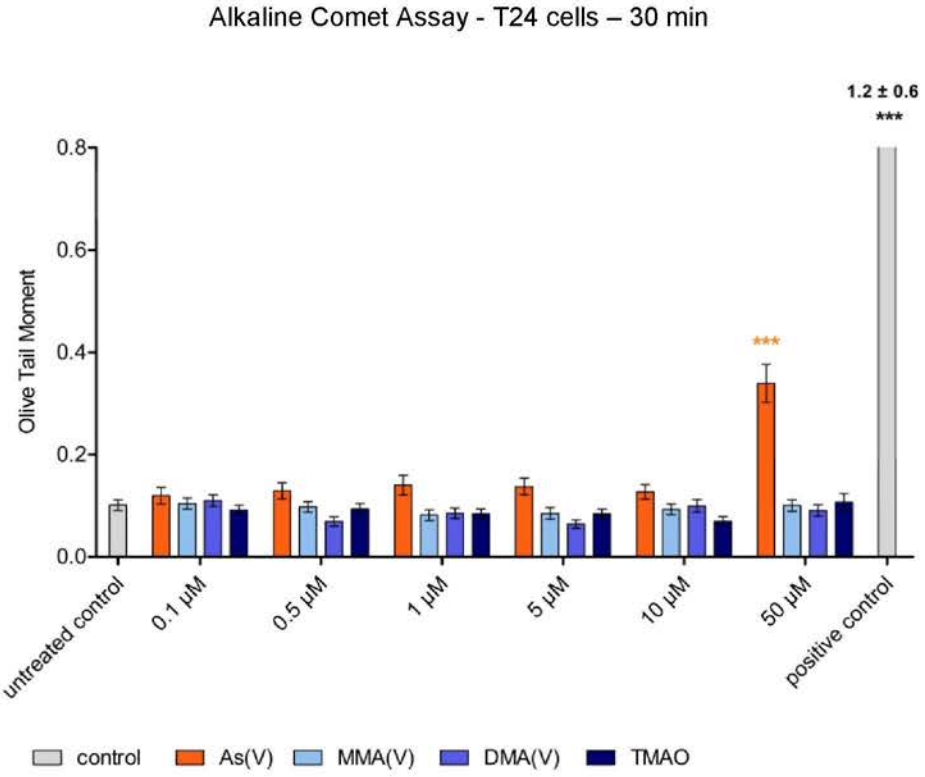


Fig. 36 DNA damage in T24 cells after 30 min of treatment with pentavalent arsenic species
The Alkaline Comet Assay was conducted to assay single and double strand breaks of the DNA after exposure to As(V), MMA(V), DMA(V) and TMAO. The DNA damage is given in the Olive Tail Moment
(mean ± SEM with p > 0,05: non-significant, p ≤ 0,05: *, p ≤ 0,01: **, p ≤ 0,001: *; n = 150)**
(Zdrenka et al., 2012, modified)

Elongation of the exposure time up to 60 min did not reveal significant differences in genotoxicity except for As(V), which seemed to cause no DNA damage any more (for details please refer to Appendix 7.2, Tab. 10). Slight cytotoxicity was observed during microscopic evaluation, and hence, this effect is considered to be preparation based.

A comparison of MMA(III)-induced genotoxicity in T24, HepG2, and HUEPC cells revealed that T24 cells were least susceptible of all. In contrast, HUEPC cells exhibited the strongest sensitivity against DNA breakage after 30 min of exposure (Fig. 37). In addition, notably reduced cell numbers were observed in treated HUEPC cells. The numbers given in the respective columns represent the number of evaluated cells of each sample. In both T24 and HepG2 samples more than 150 cells were evaluable, whereas in HUEPC cells only 6 (50 µM MMA(III)) up to 41 cells (0.5 µM MMA(III)) could be analysed, highlighting the strong cytotoxicity observed in HUEPC cells. This notice is in concordance with the observations and results presented above.

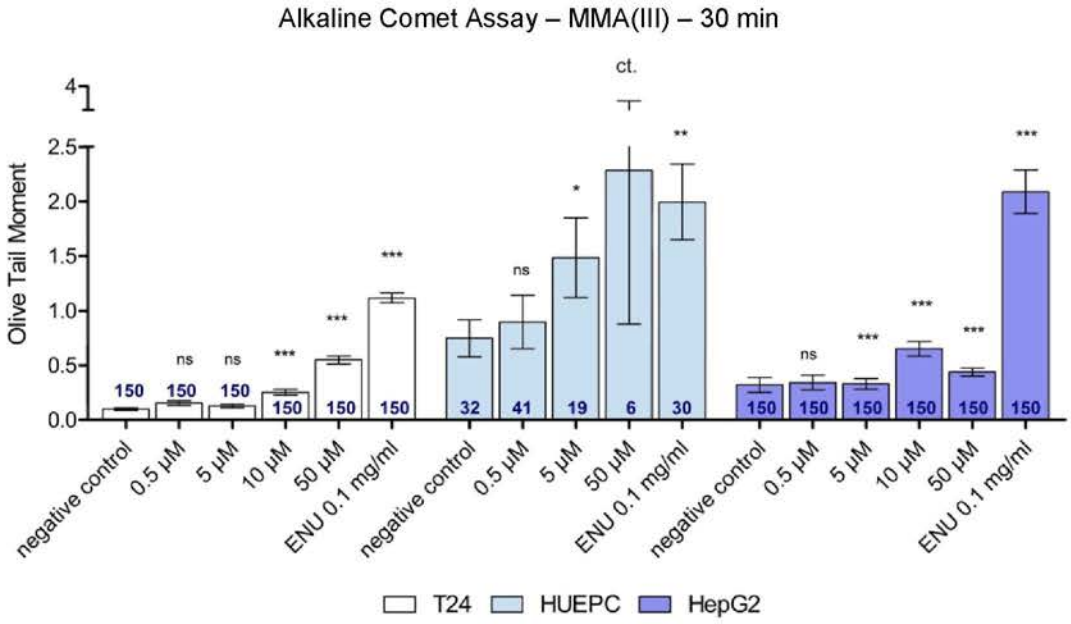


Fig. 37 DNA damage in T24, HepG2, and HUEPC cells after 30 min of treatment with MMA(III)

The Alkaline Comet Assay was conducted to assay single and double strand breaks of the DNA in T24, HepG2, and HUEPC cells after exposure to MMA(III). The DNA damage is given in the Olive Tail Moment (mean ± SEM with $p > 0,05$: non-significant, $p \leq 0,05$: *, $p \leq 0,01$: **, $p \leq 0,001$: *, the numbers of evaluated cells are given in the columns.)**

4.3 COX-2 activation

Among acute cytotoxic and genotoxic effects, especially at high doses, arsenic also exhibits tumour-promoting activities after chronic low-dose exposure. As one of the manifold molecular markers during carcinogenesis, mRNA and protein levels of COX-2 (official gene name PTGS2), were measured in this study.

Fig. 38 presents COX-2 protein levels in T24 cells after short-term exposure up to 1 week. MMA(III) induces distinct decreased COX-2 protein levels as compared to the concurrent untreated negative control at both concentrations of 75 and 100 nM after 1 h of exposure, whereas the levels normalised again within 24 h. Already after 1 week of exposure elevated COX-2 protein levels were detected in both 75 and 100 nM MMA(III)-treated cells.

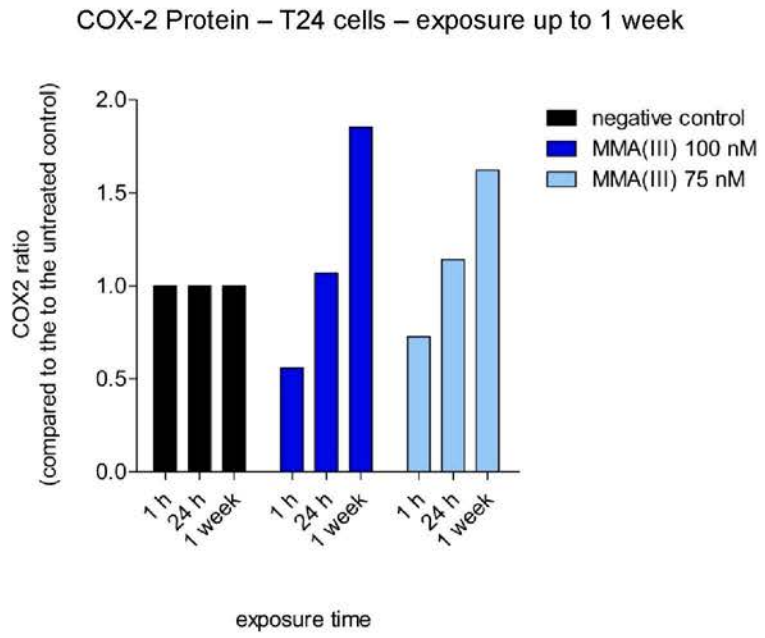


Fig. 38 COX-2 protein levels in T24 cells

The ELISA assay was conducted to analyse the COX-2 protein levels in T24 cells after exposure to 75 and 100 nM MMA(III) for 1 h, 24 h, and 1 week, respectively. Values are given as COX-2 ratio in treated cells as compared to the untreated negative control. Since this experiment was conducted once only, no statistical analysis was performed.

Chronic low-dose exposure of T24 cells to 75 and 100 nM MMA(III) revealed similar results (Fig. 39). Elevated COX-2 protein levels were observed for more than 75 weeks, whereas 75 nM caused an even more distinct increase than 100 nM MMA(III). Nevertheless, fluctuations were observed at both concentrations. In contrast, analysis

of the COX-2 mRNA levels did not reveal such distinct effects. Up to 32 weeks of exposure mRNA levels fluctuate in both untreated control and 100 nM MMA(III)-treated cells, finally leading to a relatively constant level slightly below the value from the beginning of the study (Fig. 40).

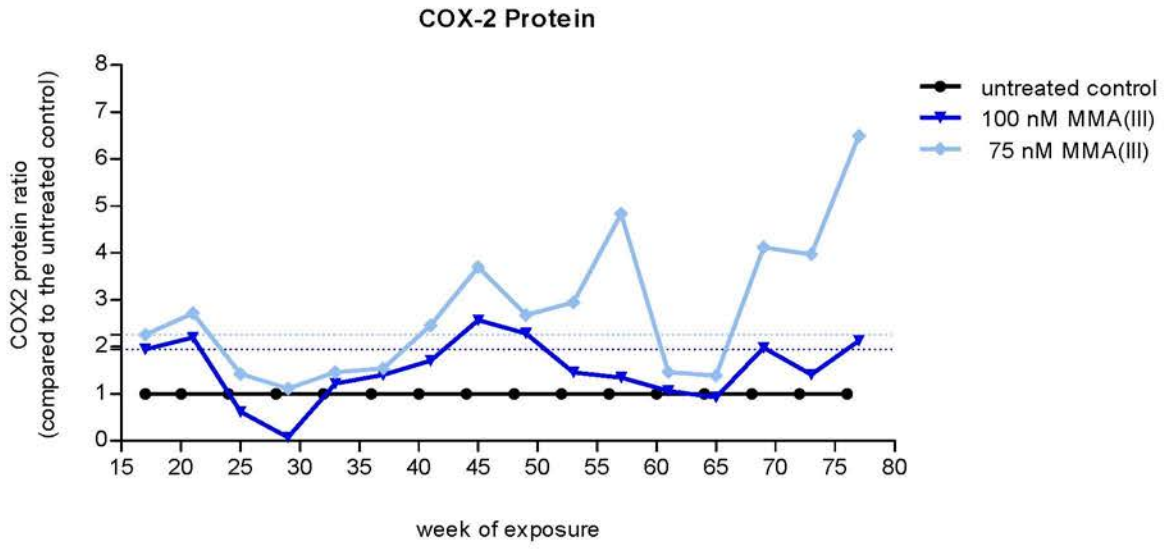


Fig. 39 COX-2 protein levels in T24 cells after chronic low-dose exposure with 75 and 100 nM MMA(III)

Values are given as COX-2 ratio in treated cells as compared to the concurrent untreated negative control. Since this experiment was conducted once only, no statistical analysis was performed.

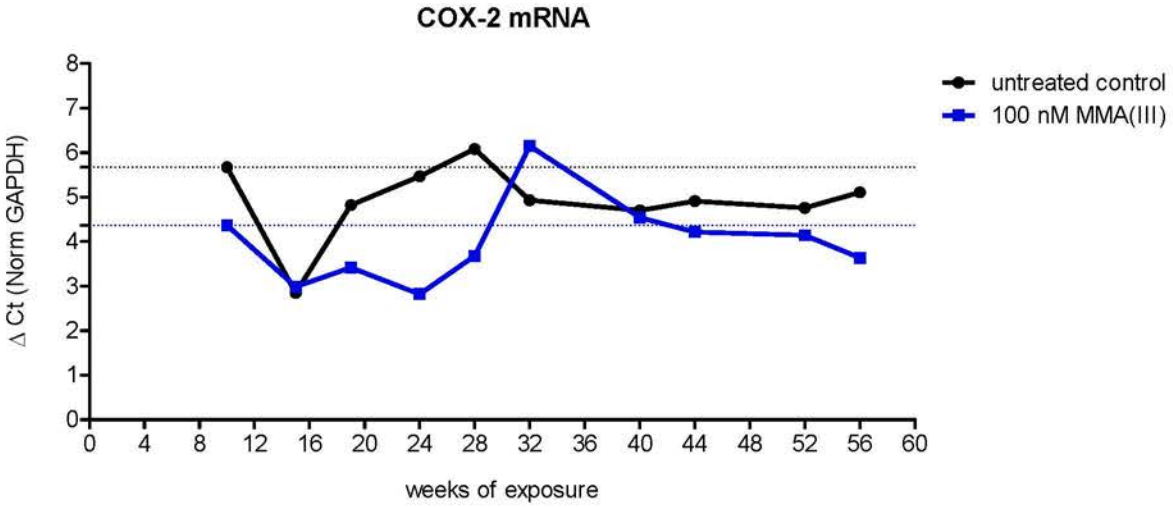


Fig. 40 COX-2 mRNA in T24 cells during chronic low-dose exposure to MMA(III) in comparison to concurrent untreated control cells.

Δ Ct values were normalised to GAPDH. Since this experiment was conducted once only, no statistical analysis was performed.

4.4 miRNA analysis

At this point I want to express my gratitude to my colleagues and project partners Dr. D. G. Weber and Dr. O. Bryk from the Institute for Prevention and Occupational Medicine of the German Social Accident Insurance (Institute of the Ruhr-University Bochum, Germany) under the head of Dr. G. Johnen, who kindly performed the experiments of the miRNA analysis and provided the data for the present study.

Results of the miRNA analysis

Altered miRNA levels were reported for several cancer diseases as they are involved in the regulation of the EMT and other processes: they are known as molecular modulators and were described as essential components of fundamental signal transduction of carcinogenesis (Dalmay & Edwards, 2006; Sotiropoulou et al., 2009). In this study miRNA analysis was performed using the TaqMan assay. 33 miRNAs were analysed in T24 cells after exposure to 100 nM MMA(III) for 10, 15, 19, 24, 28, 32, 36, 40, 44, 48, 52, and 56 weeks, respectively. The selected miRNAs were namely miR-let-7f, -7g, miR-15a, -16, 17, -18a, -19a, -19b, -20A, -26b, -30a, -92a, -96, -98, -122, -126, -128a, -140, -142, -144, -155, -182, -190, and -199a.

The following miRNAs showed significant expression alterations after arsenic axposure as compared to the concurrent negative controls: miR-15a, -19a, -19b, -30a-3p, -126, -128a, and -182 for at least 3 time points, whereas miR-let-7f, -7g, miR-16, -17, -18a, -20a, -26b, -92a, -96, -98,and -140-5p were significantly altered at only one or two time points. The remaining miRNAs (miR-122, -142-5p, -144, -155, -190, -199a-5p) were either unchanged or not detectable. The results are given in Tab. 4.

The latter 9 miRNAs of Tab. 4 were recently associated with bladder cancer (Catto et al., 2009; Dyrskjot et al., 2009; Ichimi et al., 2009; Lin et al., 2009; Veerla et al., 2009), and hence, they were analysed for all exposure concentrations (50, 75, and 10 nM) of MMA(III) from week 10 up to week 56. They all showed significantly altered expression patterns in treated cells for at least two time points or for at least two arsenic concentrations.

It is notable that from about week 36 on a global decrease of miRNAs occurs, whereas few of them even stay at the lower level up to study termination. Among those are miR-139 and miR-146a, as well as miR-200a and -429. This result was not

only observed at 100 nM MMA(III), but also at 50 and 75 nM MMA(III) and 50 nM As(III). Fig. 41 shows exemplarily the course of miR-429, the values are presented as relative changes to the untreated control. Although a first increase of miR-429 was observed, a distinct decrease of miR-429 over time is also notable from week 19 onwards.

Tab. 4 Altered expression of 33 selected miRNAs in T24 cells during chronic low-dose exposure to 100 nM MMA(III) for up to 56 weeks

A red fiels symbolises a statistically significant decrease (RQ < 0,5), anda green field shows a statistically significant increase (RQ > 2,0) of expression patterns of treated cells in comparison to untreated cells. A white field symbolises a steady expression, whereas a grey field was used when the respective miRNA was not detectable.

	10W	15W	19W	24W	28W	32W	36W	40W	44W	48W	52W	56W
let-7f												
let-7g												
miR-122												
miR-126												
miR-128												
miR-140-5p												
miR-142-5p												
miR-144												
miR-155												
miR-15a												
miR-16												
miR-17												
miR-182												
miR-18a												
miR-190												
miR-199a-5p												
miR-19a												
miR-19b												
miR-20a												
miR-26b												
miR-30a-3p												
miR-92a												
miR-96												
miR-98												
miR-139-5p												
miR-146a												
miR-149												
miR-193a-5p												
miR-200a												
miR-205												
miR-218												
miR-429												
miR-483-5p												

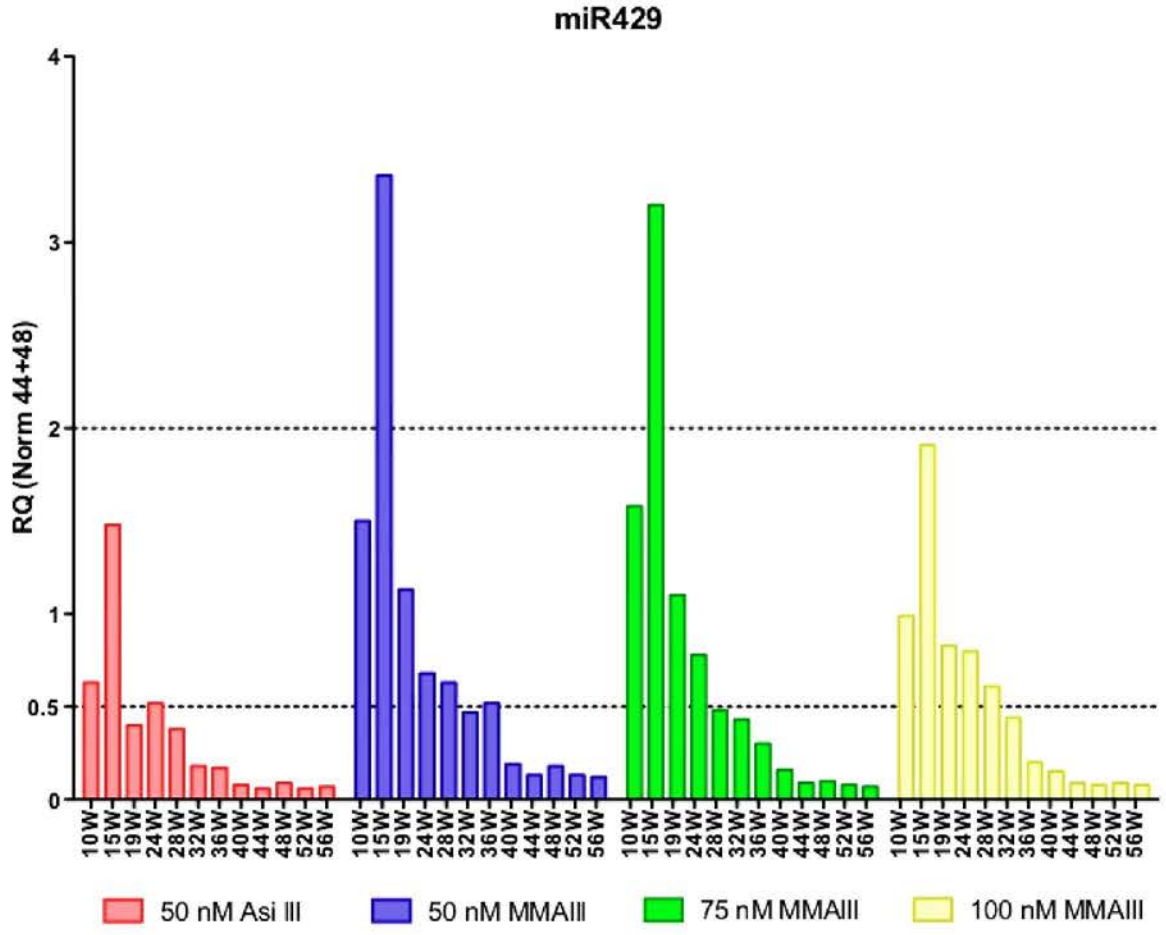


Fig. 41 Altered miR-429 expression in T24 cells after arsenic exposure up to 56 weeks
Given are the relative changes in treated cells as compared to the concurrently untreated negative controls of the same passage. Since this experiment was conducted once only, no statistical analysis was performed.

4.5 DNA Methylation

At this point I want to express my gratitude to my colleagues and project partners P. Rozynek and Y. von der Gathen from the Institute for Prevention and Occupational Medicine of the German Social Accident Insurance (Institute of the Ruhr-University Bochum, Germany) under the head of Dr. G. Johnen, who kindly performed the experiments of the DNA Methylation analysis and provided the data for the present study.

Results of the DNA Methylation analysis

Altered DNA methylation patterns were commonly reported in various cancer diseases. These alterations include both global DNA hypomethylation as well as promoter hypo and hypermethylation. While global DNA hypomethylation leads to genomic instability, promoter hypomethylation can result in oncogene activation and promoter hypermethylation might lead to the inactivation of tumour suppressor genes. Since DNA methylation is such an unique mechanism of epigenetic regulation, whose alteration can have fatal consequences such as cancer development, the study addresses DNA methylation analysis of several genes known to be involved in carcinogenesis.

As a first step, a baseline was established for the methylation status of T24 cells. Therefore, a change in the methylation status of T24 cells in contrast to non-tumorigenic urothelial cells was noted. T24 cells showed a decreased methylation of LINE1 (45% instead of approx. 75%), whereas the following genes, which are usually unmethylated in primary urothelial cells, showed a distinct increase of DNA methylation: DAPK1 (20%), RAR ß (60%), CDH1 (22%), and RASSF1 (90%). In contrast, FHIT, MGMT, and SOCS3 did not show any altered promoter methylation patterns. These alterations were considered to be the result of the immortalization of this cell line.

When the T24 cells were cultured for 68 weeks without arsenic treatment, their methylation patterns were observed to be stable in the seven genes described above (Fig. 42 to Fig. 47). At the same time, the treated cells showed none or only minor alterations (Fig. 42 to Fig. 45). Only for C1QTNF6 (Fig. 46) and CDH1 (Fig. 47) an increase of methylation over time form 22% to 35% was observed, whereas this development only started at approx. week 36 of exposure. This observation is in accordance with the fact that expression of miRNAs, which are known to be responsible for the suppression of metastasis and invasion, were decreased at the same time.

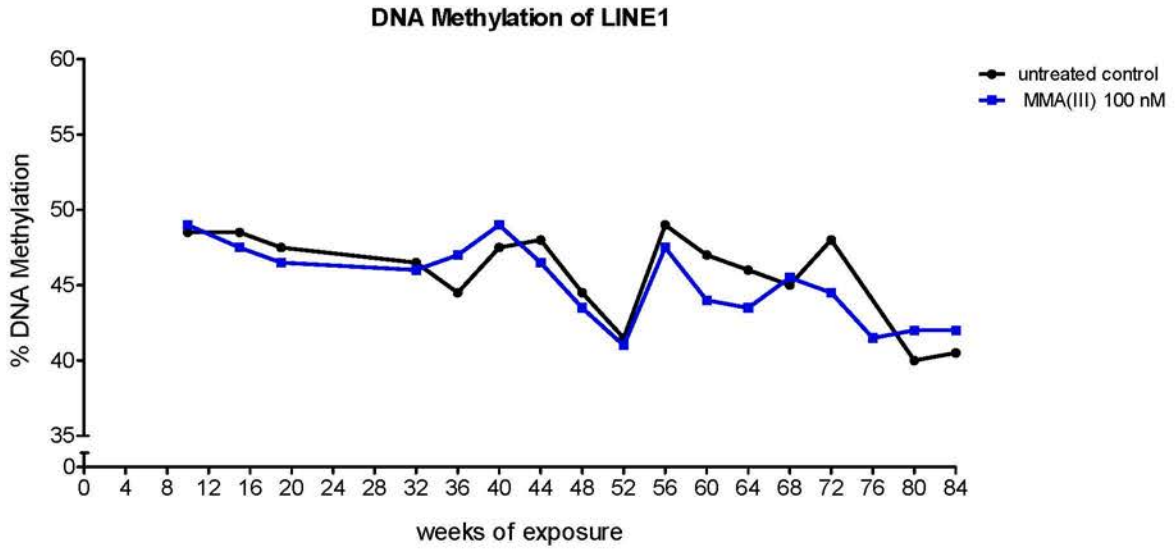


Fig. 42 Methylation pattern of LINE1 in cultured T24 cells with and without (control) chronic low-dose treatment to 100 nM MMA(III)

Since this experiment was conducted once only, no statistical analysis was performed.

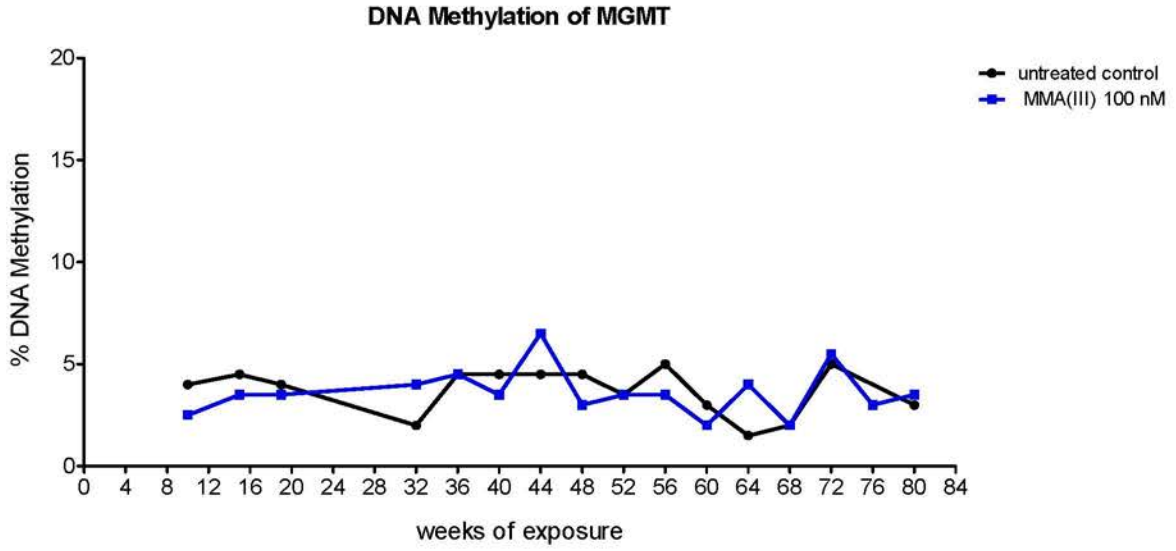


Fig. 43 Methylation pattern of MGMT in cultured T24 cells with and without (control) chronic low-dose treatment to 100 nM MMA(III)

Since this experiment was conducted once only, no statistical analysis was performed.

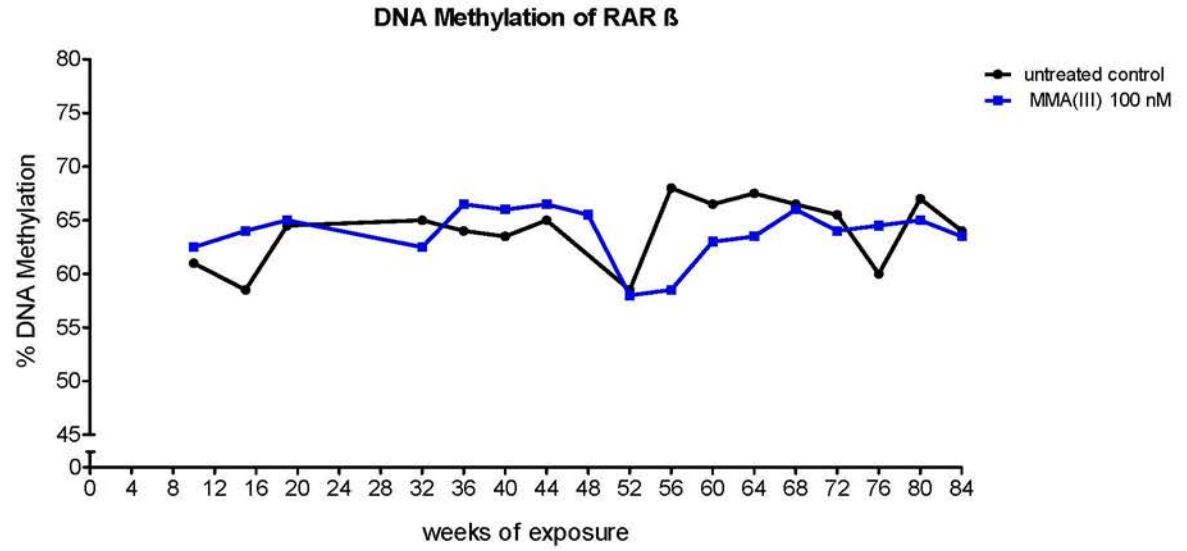


Fig. 44 Methylation pattern of RAR ß in cultured T24 cells with and without (control) chronic low-dose treatment to 100 nM MMA(III)

Since this experiment was conducted once only, no statistical analysis was performed.

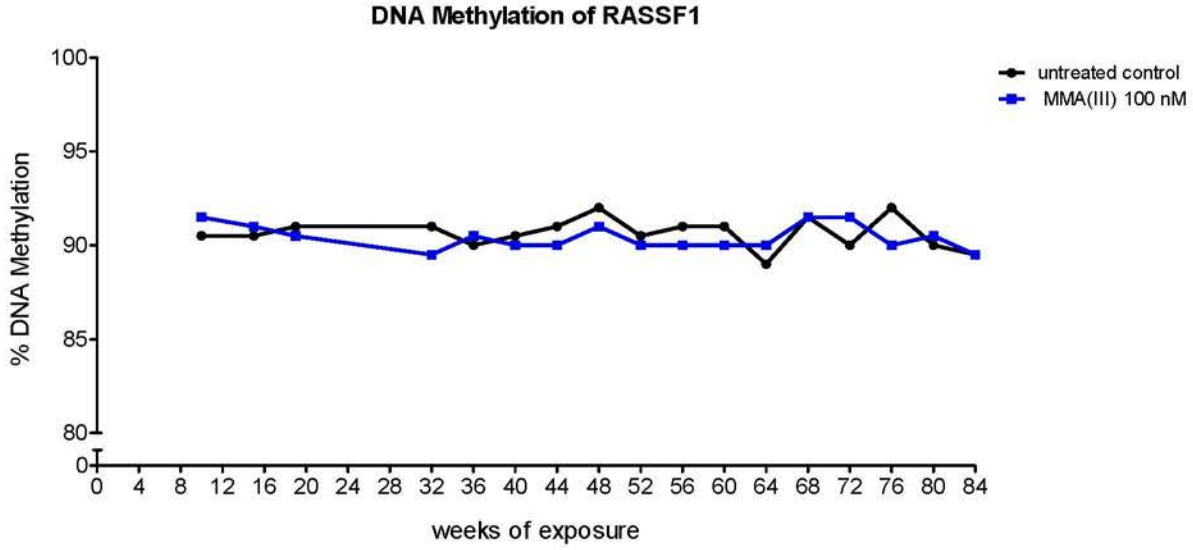


Fig. 45 Methylation pattern of RASSF1 in cultured T24 cells with and without (control) chronic low-dose treatment to 100 nM MMA(III)

Since this experiment was conducted once only, no statistical analysis was performed.

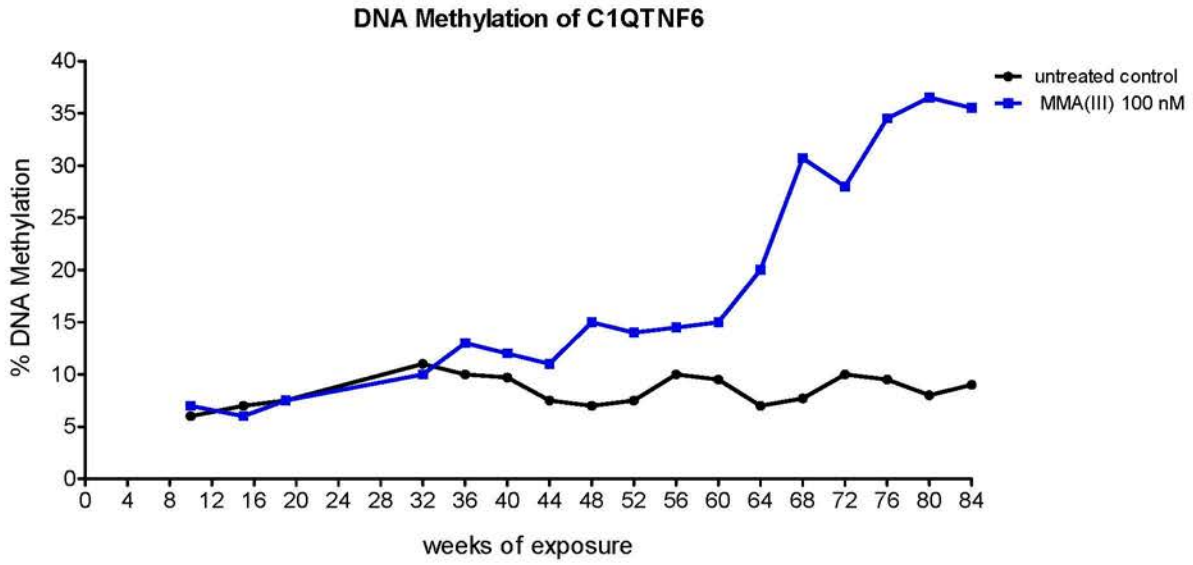


Fig. 46 Methylation pattern of C1QTNF6 in cultured T24 cells with and without (control) chronic low-dose treatment to 100 nM MMA(III)

Since this experiment was conducted once only, no statistical analysis was performed.

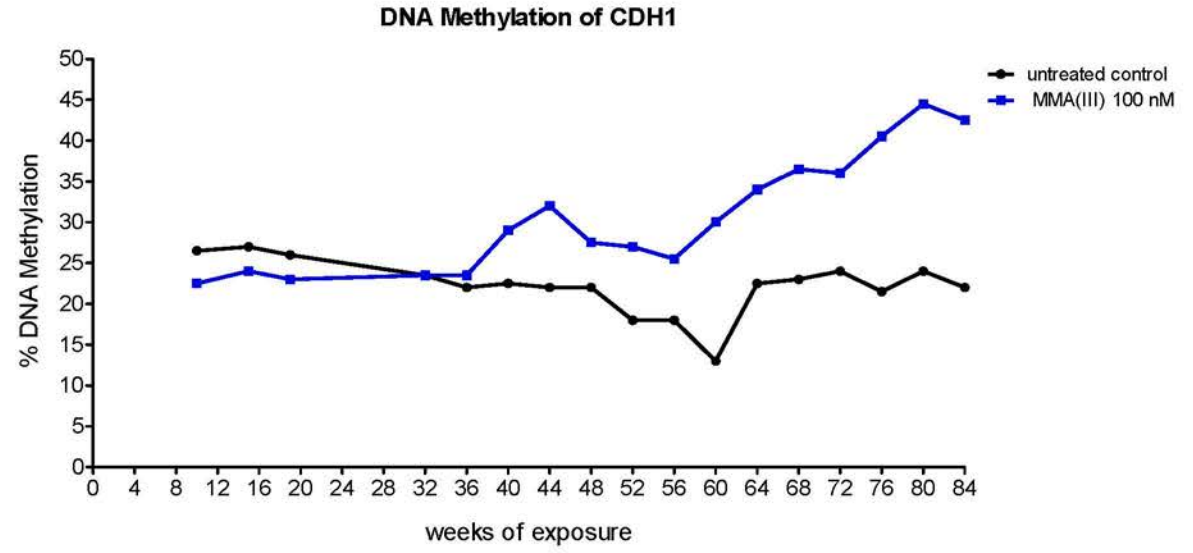


Fig. 47 Methylation pattern of CDH1 in cultured T24 cells with and without (control) chronic low-dose treatment to 100 nM MMA(III)

Since this experiment was conducted once only, no statistical analysis was performed.

The results observed for 100 nM MMA(III) treatment were similar to those seen in T24 cells treated with 75 nM MMA(III). Fig. 48 shows the methylation patterns of CDH1, MGMT, and LINE1 in untreated T24 cells in comparison to those in T24 cells after exposure to 100 nM and 75 nM MMA(III).

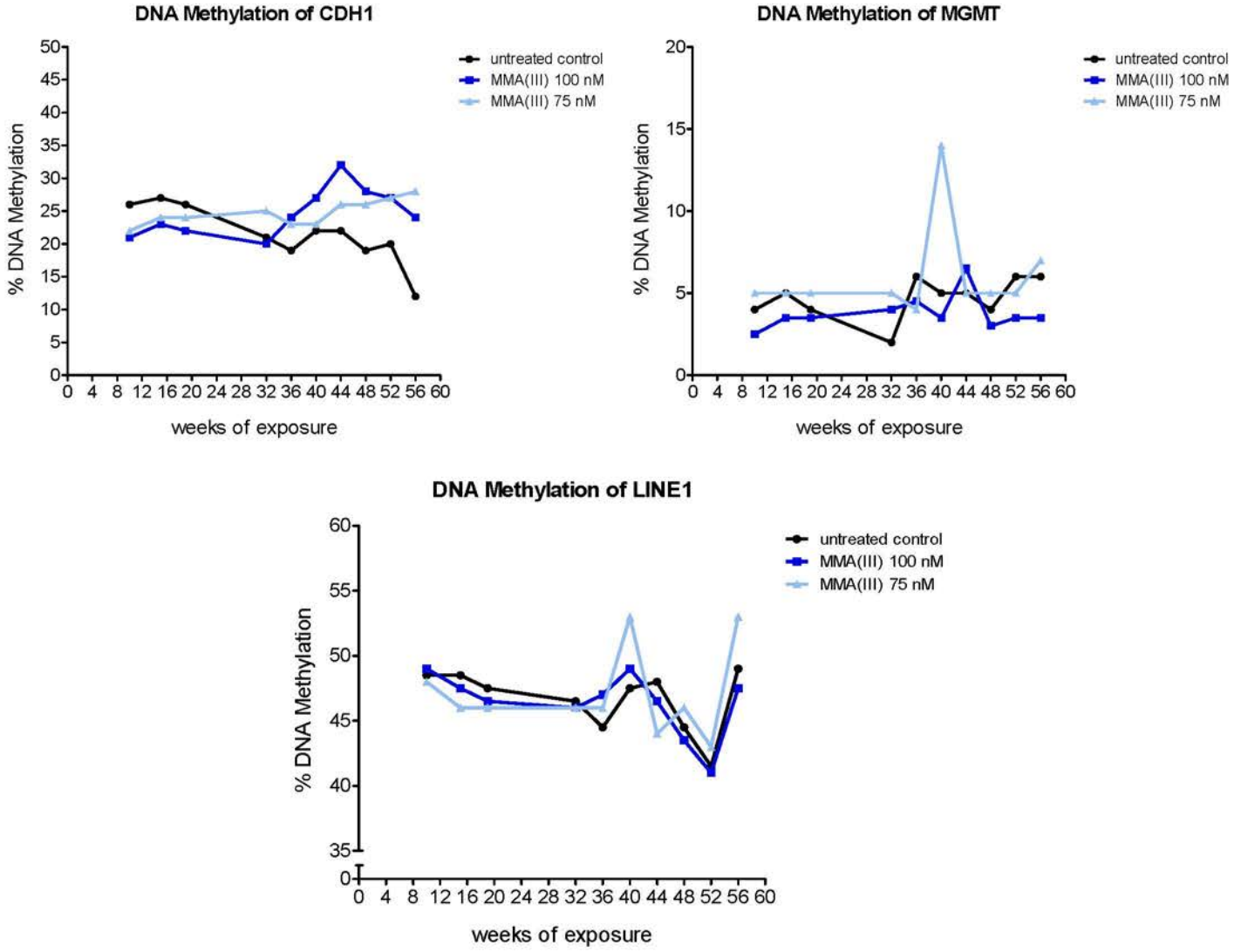


Fig. 48 Comparison of the methylation pattern of CDH1, MGMT, and LINE1 in cultured T24 cells without treatment (control) and after chronic low-dose exposure to 75 and 100 nM MMA(III)
Since this experiment was conducted once only, no statistical analysis was performed.

In addition to the genes mentioned above, two more relevant genes discussed in the current literature were analysed. Methylation of KRT7 und G0S2 during arsenic exposure was reported to increase with time (Jensen et al. 2009). However, this effect was not observed under the conditions of this study (data not shown).

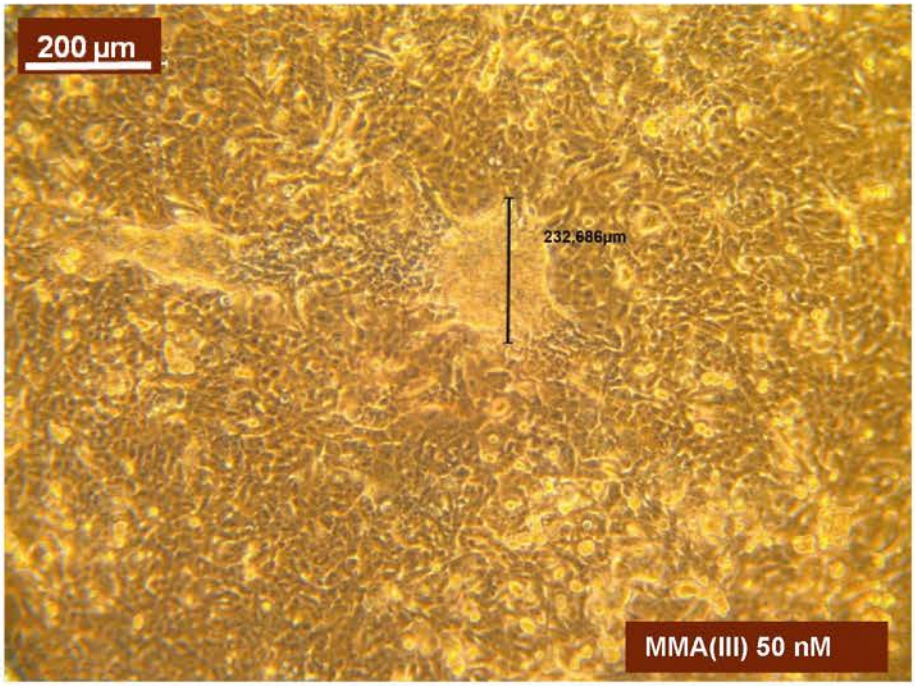
4.6 In vitro Mammalian Cell Transformation Test

The *in vitro* Mammalian Cell Transformation Test was determined to assay the loss of contact inhibition and morphological changes in chronically treated T24 cells as commonly observed phenotypic alterations during carcinogenesis. Although this cell line is known to be tumorigen, it still shows contact inhibition when cultured *in vitro* (Fig. 49). The aim of this test was to detect MMA(III) as a possible tumour promoter.

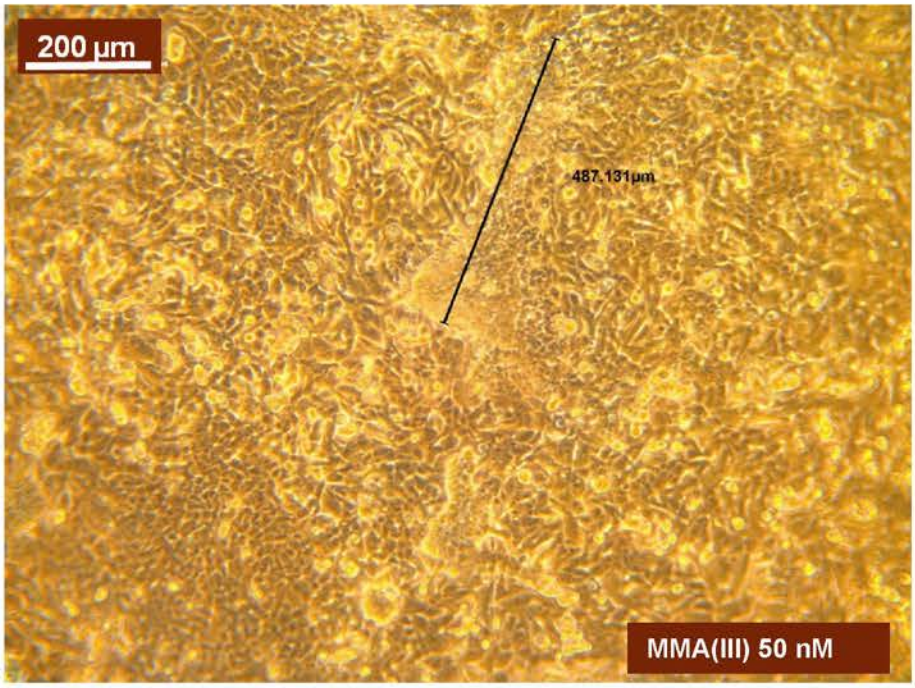
T24 cells were cultured for 92 weeks and treated twice a week with fresh exposure medium containing 50 nM, 75 nM and 100 nM MMA(III), respectively. Untreated T24 cells of the same passage served as negative control. A confluent monolayer of cells from each exposure group was incubated for another 2 weeks and subsequently visually evaluated for treatment related effects (Fig. 49).

It is obvious that chronic exposure to MMA(III) at environmentally relevant doses revealed distinct effects on T24 cells as compared to concurrently untreated negative control cells. Morphological changes have developed at all doses, including misshapen and over-sized cells, whereas cells treated with 50 and 75 nM MMA(III) exhibited the strongest effects. Moreover, distinct increases in colony formation due to the loss of contact were observed at both 50 and 75 nM doses. Such treated T24 cells formed colonies with a diameter of more than 200 µm. However, only few barely perceptible colonies were built up after treatment with 100 nM MMA(III).

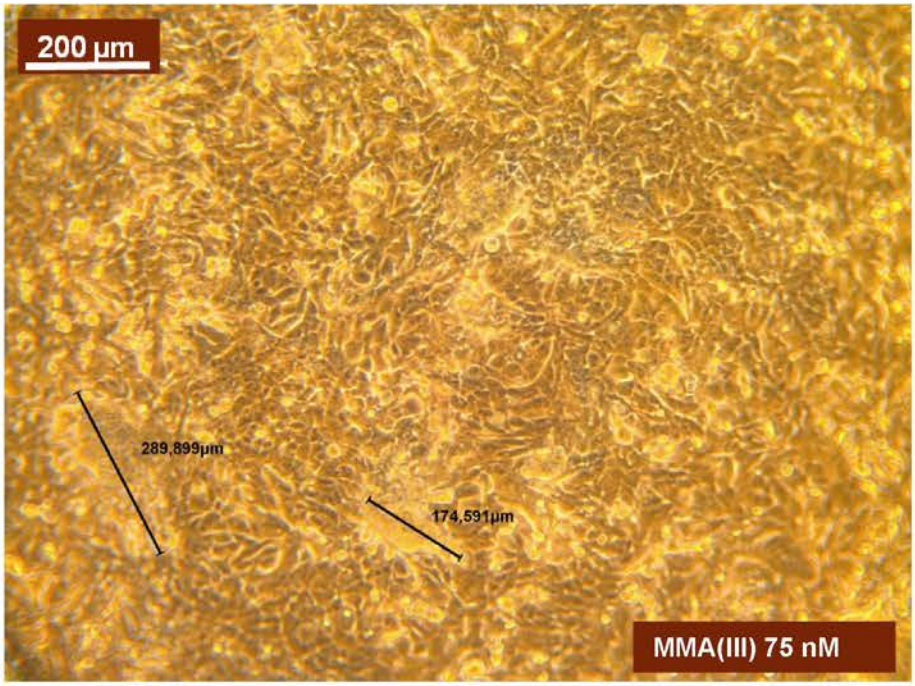
(A)



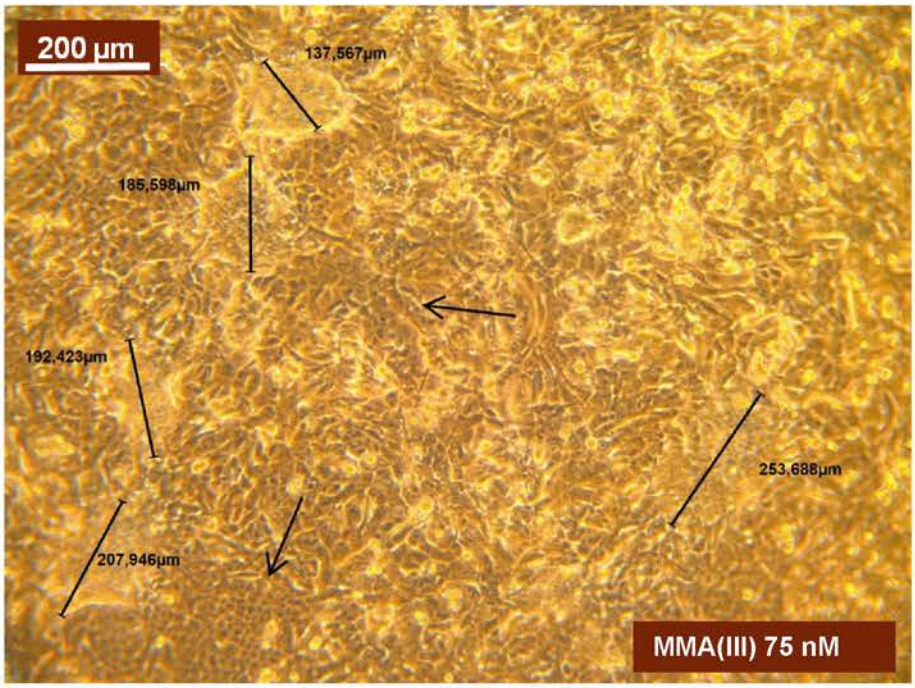
(B)



(C)

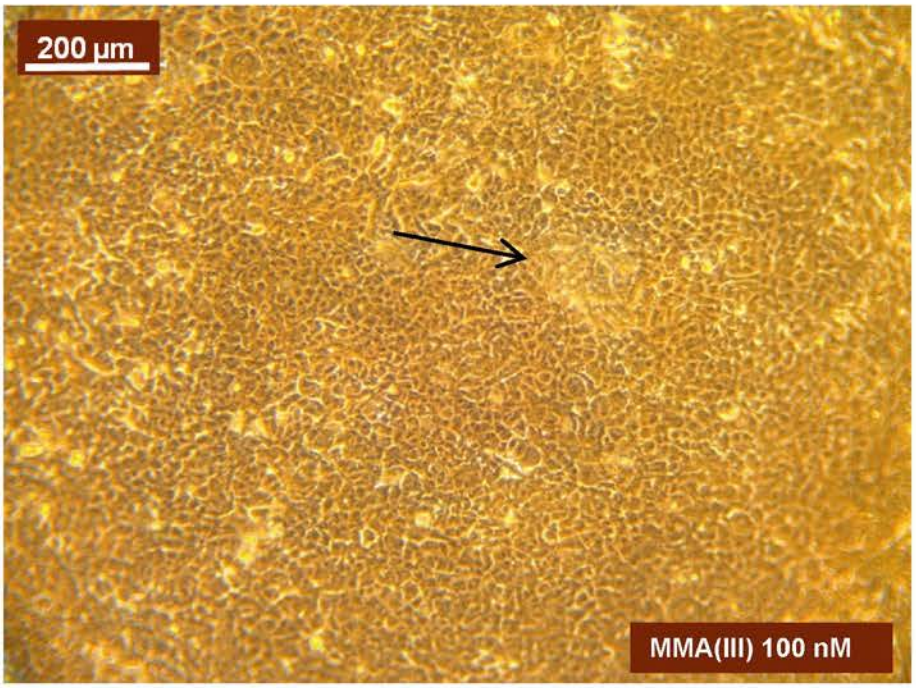

200 µm
289,899µm
174,591µm
MMA(III) 75 nM

(D)

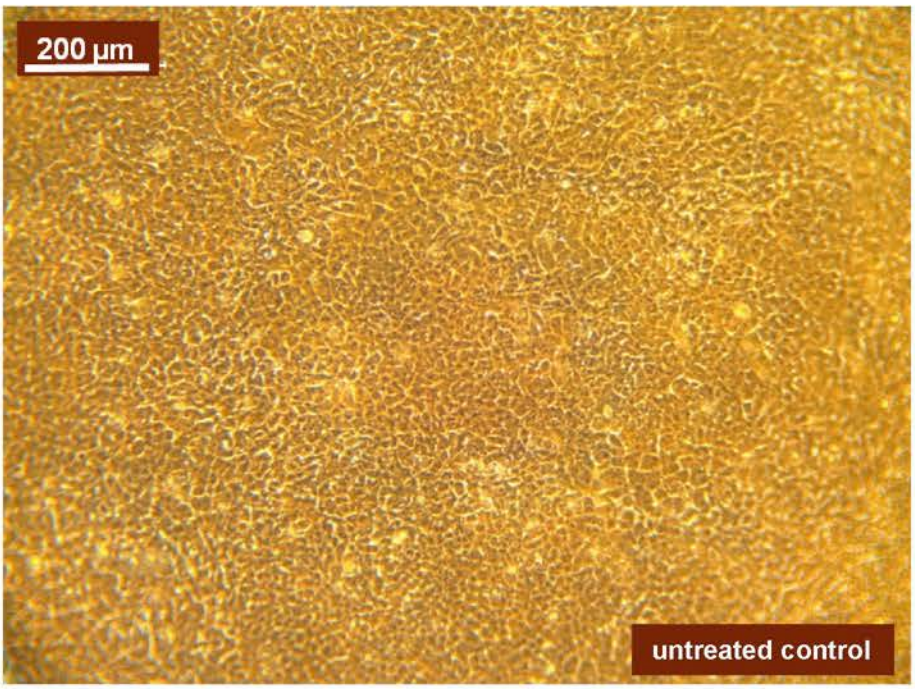

200 µm
137,567µm
185,598µm
192,423µm
253,688µm
207,946µm
MMA(III) 75 nM

(E)

(F)



(G)



(H)

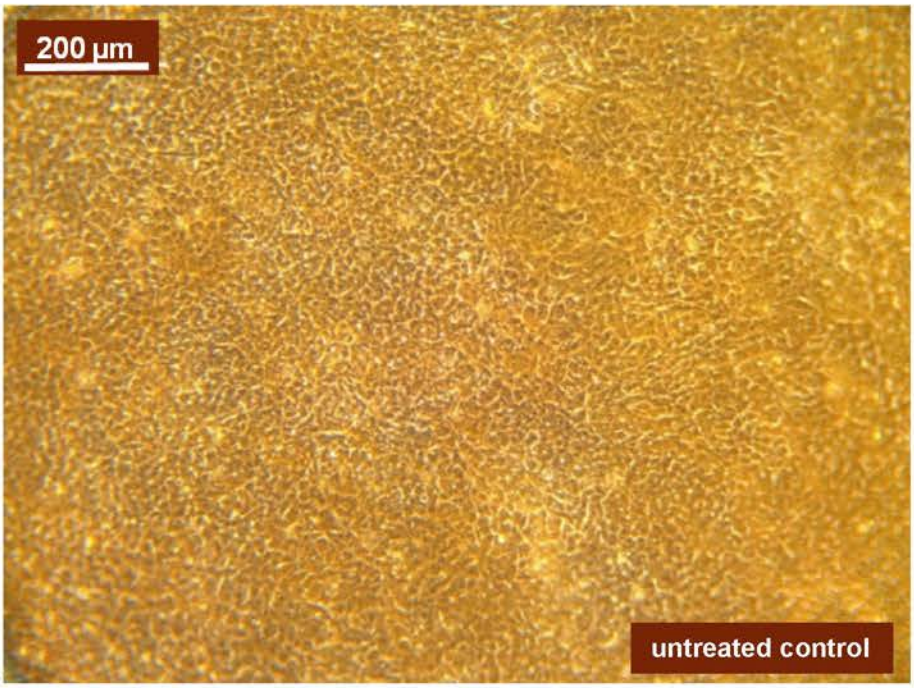
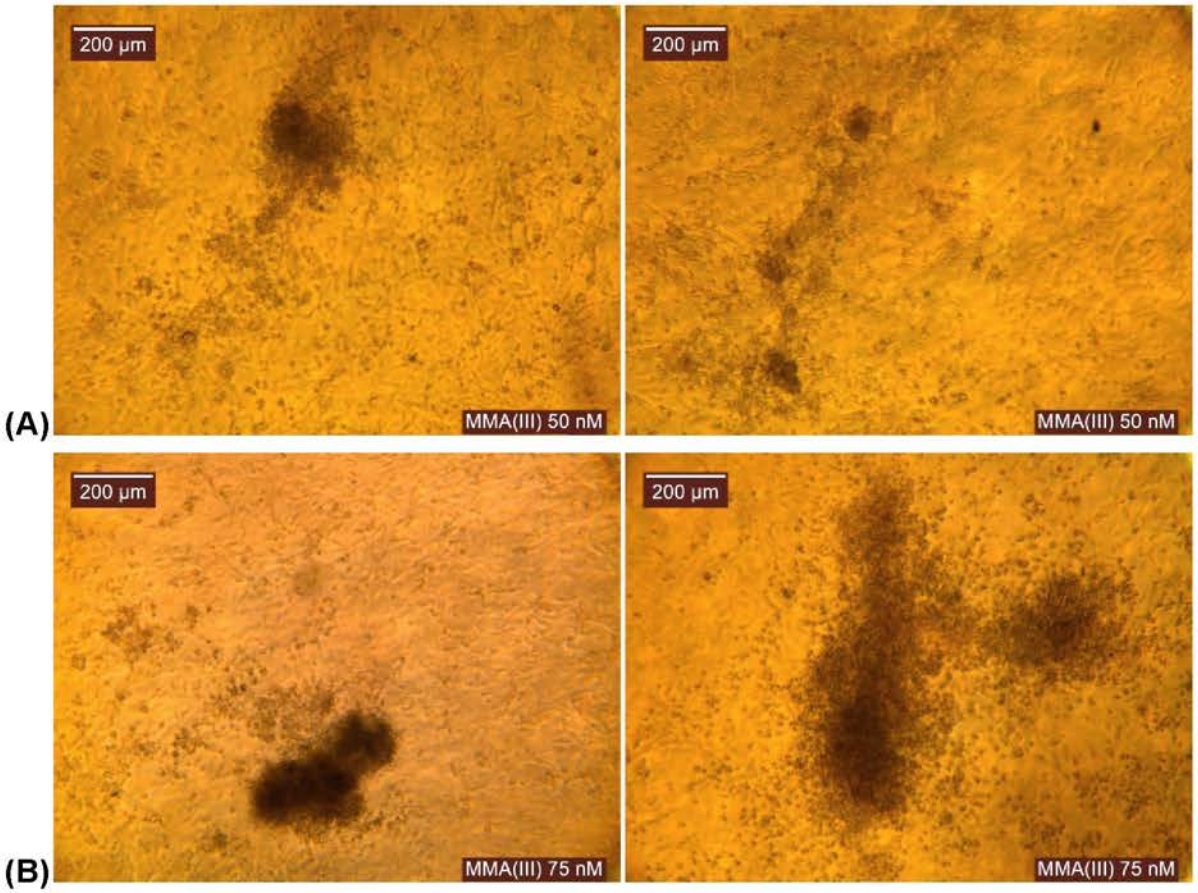


Fig. 49 *In vitro* Mammalian Cell Transformation Test with T24 cells after chronic exposure to MMA(II)

The *in vitro* Mammalian Cell Transformation Test was conducted for the analysis of morphological changes and the loss of contact inhibition after chronic treatment of T24 cells with 50 nM (A+B), 75 nM (C+D), or 100 nM (E+F) MMA(III), respectively, for 92 weeks. The concurrent negative control (G+H) consisted of untreated T24 cells of the same passage. Given are two pictures of parallel treated samples.

4.7 Colony Formation Assay

The Colony Formation Assay was determined to assay the loss of anchorage dependent growth. Therefore, T24 cells were cultured for 93 weeks and treated twice a week with fresh exposure medium containing 50 nM, 75 nM and 100 nM MMA(III), respectively. Untreated T24 cells of the same passage served as concurrent negative control. The cells were bedded into soft agar and incubated for two weeks. Although T24 cells are tumorigen, they cannot be cultured in soft agar, which was proven by the negative control. In contrast, after chronic exposure to MMA(III) T24 cells exhibited an anchorage-independent growth and formed notable colonies (Fig. 50). The colonies observed are tumour-like, and especially at a concentration of 75 nM clearly defined colonies were observed. The colonies formed of T24 cells treated with 50 and 100 nM MMA(III), respectively, were smaller in size than those of 75 nM-treated cells (Zdrenka et al., 2012).



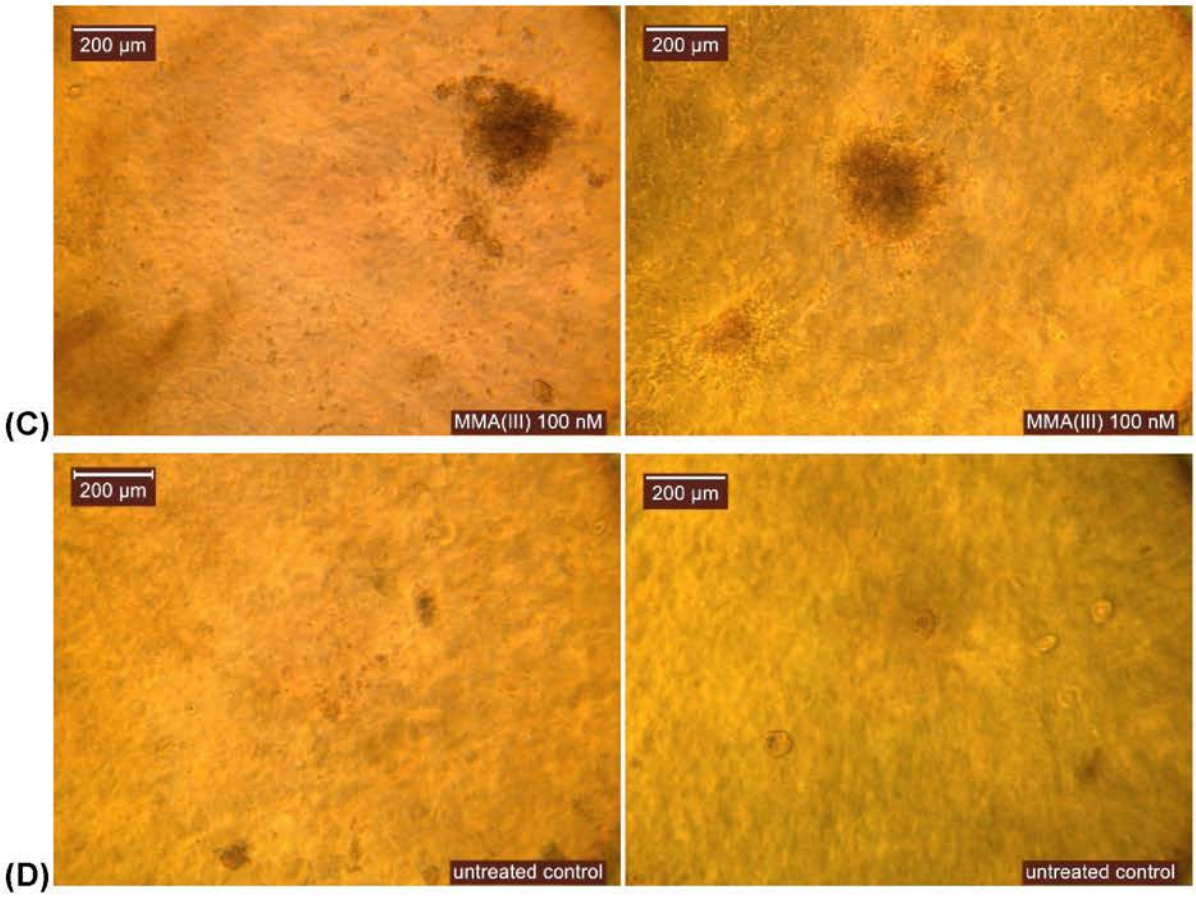


Fig. 50 Colony Formation Assay with T24 cells after chronic exposure to MMA(II)

The Colony Formation Assay was conducted for the analysis of an anchorage-independent growth after chronic treatment of T24 cells with 50 nM (A), 75 nM (B), or 100 nM (C) MMA(III), respectively, for 93 weeks. The concurrent negative control (D) consisted of untreated T24 cells of the same passage. Given are two pictures of parallel treated samples (Zdrenka et al. ,2012).

4.8 Cellular migration and invasion

First data were already presented and discussed in Zdrenka et al. (2012), wherefrom the respective information was recited in the following chapter.

To estimate the increase of malignancy of chronically arsenic-exposed human urothelial cells, their altered motility and invasiveness were examined. The xCELLigence system was conducted to assay the migration and invasion of T24 cells after 92 weeks of exposure to MMA(III). Hereby the cells moved through a microporous membrane and attached at the opposite side on the electrodes. For the analysis of the invasiveness the membranes and electrodes were coated with collagen, a typical extracellular matrix. The results show, that after exposure to 75 and 100 nM MMA(III) for 92 weeks the motility of T24 cells was increased in comparison to the concurrently untreated control of the same passage. Only the

exposure to 50 nM MMA(III) did not lead to an increase of migrated cells (Fig. 51) (Zdrenka et al., 2012).

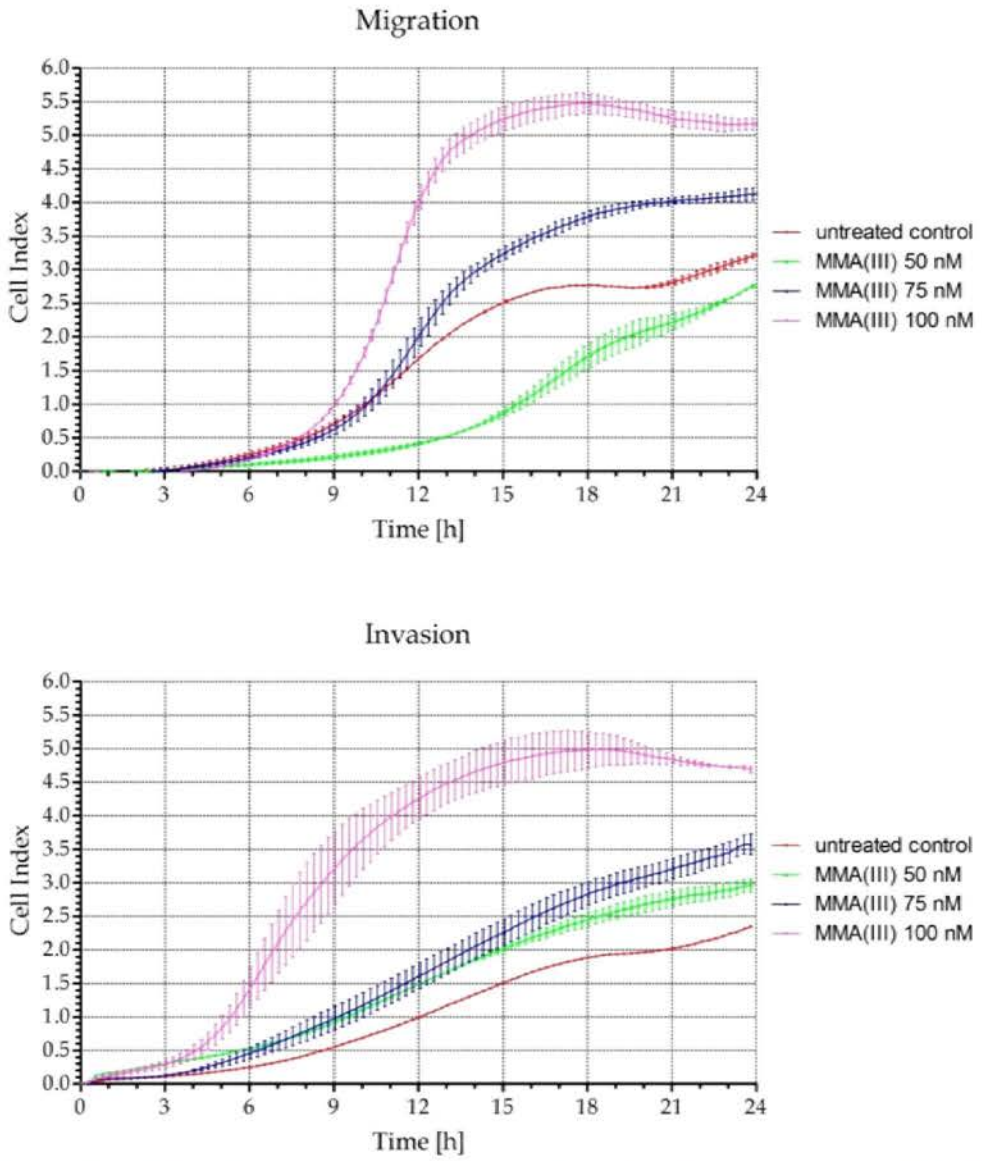


Fig. 51 Migration (A) and Invasion (B) of T24 cells after chronic exposure to MMA(II)

The xCELLigence system was conducted for the analysis of the ability of migration and invasion after chronic treatment of T24 cells with 50, 75, or 100 nM MMA(III), respectively, for 92 week. CIM-Plates were coated with collagen for invation experiments (B) to simulate a biological matrix (Zdrenka et al. ,2012). Since this experiment was conducted once only, no statistical analysis was performed.

The examination of the invasion led to similar results. After coating the plate surface with collagen all samples exhibited an increased invasion property as compared to the concurrently untreated control. In both migration and invasion assays the cells treated with 100 nM MMA(III) exhibited the strongest effects, leading to the assumption of a dose-dependent manner (Zdrenka et al, 2012).

5 Discussion

Arsenic is one of the most harmful toxins in drinking water worldwide. At many places on earth the arsenic concentrations found are 10 µg/l and above, clearly exceeding the tolerable value recommended by the WHO (WHO, 2001). Moreover, human exposure in daily life also occurs via food (fish, rice, etc.) and smoke, as well as occupationally (Food Standards Agency of th UK, 2004; Lozna & Biernat, 2008; Meharg et al., 2008; Signes-Pasto et al., 2009; Sun et al., 2009). Hence, at many places on earth millions of people are at risk of arsenic-induced diseases such as cancer. Several kinds of arsenic-induced cancer diseases have already been reported, including skin, lung, kidney, and bladder cancer (Chiou et al., 1995; Tseng, 2007; Chen et al., 2010; Ren et al., 2011).

After oral uptake, arsenic might be subjected to the first-pass effect, which is a well-known metabolic pathway observed for several xenobiotics, and which is of great importance in the assessment of drug-metabolism. Orally uptaken xenobiotics are resorbed through the intestinal mucosa into the circulation, and then transported through the portal vein (Vena portae hepatis) to the liver where they finally will be subjected to hepatic metabolism (Forth et al., 2009). In the case of arsenic compounds, fast hepatic metabolism of inorganic arsenic will result in well-characterised methylated species, which are then systemically available. Hence, the present work focussed especially on methylated metabolites such as MMA(III), as these are known to be of high importance regarding systemic toxicity and carcinogenicity as compared to the parent inorganic species (As(III) or As(V)).

In the present study the cellular uptake and biotransformation of arsenic species in non-methylating human urothelial cells (T24) and methylating human hepatic cells (HepG2) was investigated by using HPLC-ICP / MS technique. The induced genotoxic effects of several arsenic species (namely As(III), As(V), MMA(III), MMA(V), DMA(III), DMA(V), and TMAO) in T24 cells were measured by means of the Alkaline Comet Assay, and the effects on malignant cell behaviour after chronic arsenic treatment was assayed, using the following *in vitro* approaches: the *in vitro* Mammalian Cell Transformation Test, the Colony Formation Assay, and the Migration and Invasion Assay. Some of the key processes altered in T24 cells by chronic arsenic exposure were analysed this study; the endpoints addressed were:

alterations in COX-2 activation, miRNA expression, and DNA methylation patterns. The present work aims to analyse the basic key events during the development of arsenic-induced bladder cancer *in vitro*.

5.1 Intracellular arsenic speciation and quantification

At this point I want to express my gratitude to my colleague and project partner J. Hippler from the Institute of Environmental Analytical Chemistry (University of Duisburg-Essen, Germany) under the head of Prof. Dr. Hirner, who kindly performed the experiments of the intracellular arsenic speciation and quantification using HPLC-ICP/MS and provided the data for the present study.

First data were already published in Hippler et al. (2011), and further results were presented and discussed in Zdrenka et al. (2012), wherefrom the respective information was recited in the following chapter.

Discussion of the intracellular arsenic speciation and quantification

The data presented here demonstrate that MMA(III) is rapidly taken up by human urothelial cells (T24) and human hepatic cells (HepG2). MMA(III) is known to be an important arsenic metabolite due to its high toxicity. Additionally, many studies report monomethylated and dimethylated arsenic species as the main metabolites in the urine (Aposhian et al., 2000; Fillol et al., 2010, as cited in Zdrenka et al., 2012). The use of an improved isolation method and HPLC-ICP/MS technique (Hippler et al., 2011) enabled the analysis of fast MMA(III) association to large membrane structures, high-molecular-weight proteins, and other insoluble cell components, and furthermore, the recovery of unbound pentavalent arsenic metabolites in the soluble fractions; these were found to amount to only 0.003% of the total intracellular arsenic in T24 cells and 0.01% in HepG2 cells. Furthermore, differentiation between the various methylated metabolites and their oxidation state in complex cellular matrices was possible (Zdrenka et al., 2012).

The results also revealed a fast cellular uptake of MMA(III) for both the T24 and HepG2 cell lines, followed by rapid oxidation to MMA(V) already within 5 min, which further increased with time (Fig. 34). Additionally, in HepG2 cells a time dependent methylation to DMA(V) was observed. These findings appear to be in contrast to the

reductive intracellular milieu (Du et al., 2009, Hippler et al., 2011, as cited in Zdrenka et al., 2012) due to glutathione concentrations up to millimolar ranges (Anderson, 1998, Hippler et al., 2011, as cited in Zdrenka et al., 2012). However, taking different cell compartments and specific metabolic effects of arsenic into account could provide a possible explanation. It is largely known from the literature that in contrast to their pentavalent analogues, trivalent arsenicals bind to proteins (Styblo & Thomas, 1997b; Yan et al., 2009, Hippler et al., 2011, as cited in Zdrenka et al., 2012). This leads to the assumption that MMA(III) rapidly binds to proteins after uptake, which is in compliance with the detection of more than 99.99% of the total arsenic in the non-soluble fraction in both cell lines (Fig. 33 and Fig. 34). A fast subsequent degradation of at least part of the arsenic-conjugated or arsenic-inhibited proteins can be presumed. Protein degradation is predominantly mediated by the proteasomes but can also take place in lysosomes, especially during turnover of membrane proteins (Clague & Urbé, 2010, Hippler et al., 2011, as cited in Zdrenka et al., 2012). An increased turnover of arsenic-bound proteins is in agreement with the evidence of the catabolism of arsenic-induced improperly folded or damaged proteins via the ubiquitin-dependent protein degradation pathway in zebrafish liver (Lam et al., 2006, Hippler et al., 2011, as cited in Zdrenka et al., 2012). Several studies report that protein ubiquitination not only targets protein degradation using the proteasome pathway but also the lysosomal pathway (Marques et al., 2004; Barriere et al.; 2007, Shenoy et al, 2008; Arancibia-Cárcamo et al, 2009, Hippler et al., 2011, as cited in Zdrenka et al., 2012). The lysosomes are known to exhibit cellular oxidative activities (Chen, 2002, Hippler et al., 2011, as cited in Zdrenka et al., 2012) and during oxidative stress large amounts of hydrogen peroxide enter the lysosome, leading to the formation of hydroxyl radicals and lysosomal destabilisation (Terman et al., 2006, Hippler et al., 2011, as cited in Zdrenka et al., 2012). In addition, the lysosome is an important organelle in autophagy, helping the cells to remove toxic aggregation-prone proteins and even damaged organelles that are incompatible with the unfolding mechanism of the proteasome (Yang et al., 2008; Clague & Urbé, 2010; Mehrpour et al., 2010, Hippler et al., 2011, as cited in Zdrenka et al., 2012). The increase of protein catabolism, the elimination of excess or damaged organelles by autophagy, and the sequestering of toxicants is ubiquitous and believed to be a kind of “first-response reaction” to delay apoptosis (Kundu & Thompson, 2008; Yang et al., 2008,

Hippler et al., 2011, as cited in Zdrenka et al., 2012). In the early stage of cell death autophagy was reported to be activated in HL60 cells soon after exposure to As_2O_3 to maintain cell survival under stress conditions (Yang et al., 2008, Hippler et al., 2011, as cited in Zdrenka et al., 2012).

Summarising all these data, it can be concluded from the results that MMA(III) is immediately taken up and rapidly bound to proteins and other cellular structures, followed by first, the generally accepted methylation to dimethylarsinic in HepG2 cells only, and second, the degradation of affected proteins and cellular structures in the lysosomes in both HepG2 and T24 cells (Fig. 52) (Hippler et al., 2011, as cited in Zdrenka et al., 2012). During oxidative degradation in the lysosomes arsenic is released as MMA(V) and DMA(V), which are either excreted from the cell or reduced by antioxidants in the cytosol. The reduced species MMA(III) and DMA(III) can then re-associate with proteins and cellular structures. In HepG2 cells most of the incorporated MMA(III) is methylated and oxidised to DMA(V) after passing this cycle; in T24 cells the cycle is limited to oxidation and reduction, and finally excretion, due to the fact that urothelial cells are non methylating (Hippler et al., 2011, as cited in Zdrenka et al., 2012).

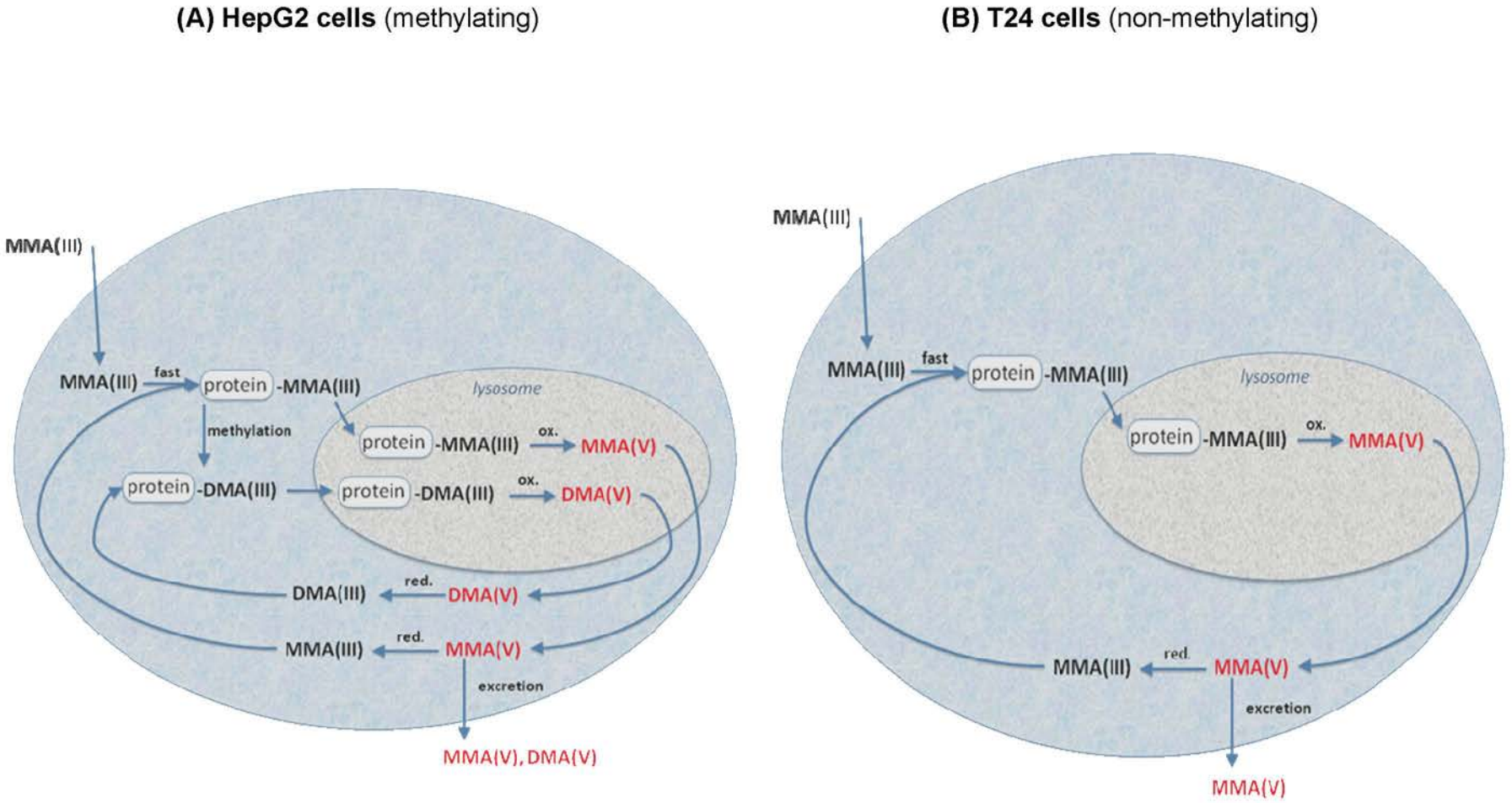


Fig. 52 Proposed arsenic cycle in HepG2 (A) and T24 cells (B) after exposure to MMA(III) (Hippler et al., 2011 (modified), Zdrenka et al. 2012)

5.2 Arsenic-induced genotoxicity

Results on arsenic-induced genotoxicity were already presented and discussed in Zdrenka et al. (2012), wherefrom the respective information was recited in the following chapter.

Especially the trivalent arsenicals are known to exhibit strong genotoxic effects (Dopp et al., 2010a, as cited in Zdrenka et al., 2012). These effects can either be detected as DNA damage using the Micronucleus Test or the Comet Assay (Dopp et al., 2008, as cited in Zdrenka et al., 2012), as oxidative damage by assaying the amount of 8-oxo-dG (8-oxo-2'-deoxyguanosine) or in form of chromosomal aberrations (Dopp et al., 2004, as cited in Zdrenka et al., 2012). In this study the Alkaline Comet Assay was utilised to analyse single and double strand breaks of the DNA in cells treated with As(III), As(V), MMA(III), MMA(V), DMA(III), DMA(V), and TMAO, respectively. Significant genotoxic effects of all tested trivalent arsenic compounds (As(III), MMA(III) and DMA(III)) and the pentavalent arsenate were detected in T24 cells already after 30 min of exposure (Fig. 35 and Fig. 36). These results are in compliance with the findings of previous studies using several mammalian cell types (Schwerdtle et al., 2003; Raisuddin & Jha, 2004; Dopp et al., 2005, as cited in Zdrenka et al., 2012). Moreover, the data presented in Fig. 37 show that the degree of genotoxicity is also dependent on the cell type and/or tissue tested. In fact, as shown in Fig. 37, immortalised cell lines such as T24 cell line, which already have undergone cell transformation to some degree, seem to be less susceptible to the genotoxic potential of arsenic compounds when compared to primary cells, such as HUEPC cells. Furthermore, the comparison of both cell lines (T24 and HepG2), clearly reveals that the kind of tissue the cells were originally derived from, is of great importance regarding the outcome of genotoxic effects. The results of the experiments conducted within the present work revealed that HepG2 cells, which originate from liver tissue, showed a greater susceptibility toward arsenic-induced genotoxicity when compared to the urothelial T24 cells. This was assumed to be related to the different metabolic capacity of the cell types, as was highlighted by the experiments dealing with the intracellular arsenic speciation. This emphasises, how important the knowledge of the tissue-dependent arsenic biotransformation as shown in Fig. 52 is, as the genotoxic effect of arsenic metabolites is not only dependent on its oxidative state, but also on the state of methylation (Hirner & Rettenmeier, 2010;

Dopp et al., 2010b, as cited in Zdrenka et al., 2012). Wnek et al. (2009, as cited in Zdrenka et al., 2012) reported that in UROtsa cells DNA damage caused by MMA(III) does not only result from acute treatment with high doses, but also from chronic exposure to low levels of MMA(III) over 12 – 52 weeks. The incidence of single- and double-strand breaks is significantly decreased after the removal of MMA(III) for 2 weeks, but still remains significantly above the untreated control. In parallel, the relative poly (ADP-ribose) polymerase (PARP) activity was noticed to be significantly reduced during this chronic exposure, and increased again after removal of MMA(III). Trivalent arsenic species are known to attach to zinc binding structures generally found in DNA repair enzymes and transcription factors, leading to alteration or inhibition of these proteins (Kitchin & Wallace, 2008). Together with the findings of Hu et al. (1998, as cited in Zdrenka et al., 2012) it is likely that DNA repair is inhibited by both, direct protein interaction and altered signal transduction or gene expression (Zdrenka et al., 2012).

Owing to all these facts, not only DNA damage itself plays a pivotal role in arsenic-induced carcinogenesic, but also inorganic arsenic and its metabolites are of importance in their function as potent epigenetic modulators, leading to (tissue specific) altered cellular functions, malignant transformation, and finally tumour development.

5.3 DNA Methylation during arsenic-induced malignancy

At this point I want to express my gratitude to my colleagues and project partners P. Rozynek and Y. von der Gathen from the Institute for Prevention and Occupational Medicine of the German Social Accident Insurance (Institute of the Ruhr-University Bochum, Germany) under the head of Dr. G. Johnen, who kindly performed the experiments of the DNA Methylation analysis and provided the data for the present study.

Discussion of the DNA Methylation during arsenic-induced malignancy

Many studies report arsenic-induced aberrant DNA methylation patterns, resulting in changes in the promoter activity that lead to altered gene expression (Sutherland &

Costa, 2003; Jensen et al., 2009; Smeester et al., 2011; Ren et al.; 2011). Aberrant DNA methylation patterns might be the result of arsenic-induced altered global histone modification, finally leading to the silencing of tumour suppressor genes (Zhou et al., 2008). Within a further experiment conducted within the present work, T24 cells were chronically treated with MMA(III) at low doses up to 100 nM. Six genes known to be involved in carcinogenesis (LINE1, MGMT, RAR ß, RASSF1, C1QTNF6, and CDH1) were exemplarily analysed for the development of their methylation status. In fact, global DNA hypomethylation could be correlated with chromosomal instability (Matsuzaki et al., 2005), and further, hypomethylation of LINE1 in particular was found to be associated with the initiation of the clonal expansion of premalignant cells in early stages of cancer development (Wilhelm et al., 2010, Zeimet et al., 2010). Therefore, the methylation status of LINE1 was first examined. In the experiment of the present work, the methylation status in T24 cells treated with 75 and 100 nM MMA(III) and in concurrently untreated control cells was found to be stable, without any treatment-related effects observed. In fact, fluctuations detected throughout the experiment were similar for both controls and treated cells as shown in Fig. 42 and Fig. 48. Hence, MMA(III)-induced effects on global DNA methylation, in particular on LINE1 hypomethylation, were not observed in T24 cells under the conditions of the test. It is quite clear that the LINE1 methylation status observed in untreated T24 control cells does not represents the status of “normal” urothelial cells, as the T24 cell line used in the present work is already a transformed tumour cell line (Johnen et al., 2013). A further influence of arsenic exposure on LINE1 methylation in existing urothelial tumour tissue seems in light of the present result unlikely.

Few data are available that human exposure to arsenic, e.g. via contaminated drinking water, leads to global DNA hypomethylation (Ren et al., 2011). Especially, the hypomethylation of LINE1 was observed in subjects suffering from bladder cancer, indicating a strong correlation between the risk of non-invasive bladder cancer and LINE1 hypomethylation. In contrast, no such strong correlation could be evidenced for invasive bladder cancer. Rent et al. (2011) suggested that LINE1 is an independent risk factor for bladder cancer and might be suitable as a biomarker. Nevertheless, a dose-response relationship between LINE1 methylation extent and the risk of bladder cancer could not yet be observed (Wilhelm et al., 2010). Thus,

further studies still are needed to assay whether LINE1 methylation patterns are directly targeted by arsenic. Altered global DNA methylation levels in general have already been correlated with the arsenic metabolism as both systems use the same methyl donor SAM (S-adenosyl-methionine). SAM is known to transfer methyl groups to DNA methyltransferases and to AS3MT (arsenic (+3 oxidation state) methyltransferase) (Ren et al., 2011, as cited in Zdrenka et al., 2012). Further studies are needed to prove this, for example by comparing methylation patterns in cells with and without metabolic capacities. Hepatic cells could be used as a kind of positive control as they are known to be methylating, while uothelial cells such as UROtsa and T24 cells lack methylation abilities. The clonal UROtsa/F35 cell line (actually T24/F35 cell line according to Johnen et al. (2013)) is known to express rat AS3MT, and therefore exhibited methylating properties (Hester et al., 2009) and might therefore serve as a link between both hepatic and urothelial cell lines.

Besides global DNA hypomethylation, promoter hypermethylation was also observed in human skin and bladder cancer, in animals *in vivo*, as well as in human and animal cell lines *in vitro* (Ren et al., 2011). In a further experiment reported in the present work, RASSF1 was analysed for MMA(III)-induced inactivation due to promoter hypomethylation. Its transcript RASSF1A was reported to exhibit tumour suppressive properties by preventing cell proliferation as a result of the inhibition of cell cycle progression through G1 into S, and the induction of cell death (Dammann et al., 2005; Burbee et al., 2011). Promoter hypermethylation of RASSF1A was observed in patients suffering from bladder cancer after arsenic exposure, and RASSF1A was further associated with invasive bladder cancer, which is the more advanced and fatal form of this disease (Dulaimi et al., 2004; Dammann et al., 2005); furthermore, a correlation between arsenic exposure, RASSF1A promoter methylation, and bladder cancer occurrence was also reported by Marsit et al. (2006b). Moreover, arsenic exposure resulted in promoter hypermethylation of RASSF1A in A/J mice and was associated with the development of lung cancer (Ren et al., 2011). In the present work chronic treatment of T24 cells with 75 and 100 nM MMA(III) did not alter promoter methylation of RASSF1 as compared to the concurrently untreated control (Fig. 45 and Fig. 48). Furthermore, no distinct fluctuations were observed in the methylation status of this promoter in neither of the test groups except for the 75 nM MMA(III)-treated culture after 40 weeks of exposure. Nevertheless, this observation

was considered to be an outlier value without any biologically relevance rather than a treatment related effect, since it was reversible and only seen at the time point and test concentration mentioned above. At a first glance the data indicate that the loss of the tumour suppressive properties of RASSF1 transcripts as one of the key events during arsenic-induced malignant transformation could not be confirmed under the conditions of this test. In light of the fact that methylation of the RASSF1 promoter region of untreated T24 cells showed already a methylation baseline of approximately 90%, a further methylation seems unlikely and therefore the use of the T24 cell line in this experiment is questionable. It should be noted at this point that the study was initially based on the mistaken assumption that the cell line used was UROtsa. Based on the results observed in the present work, the identy of the cell line was challenged and only recent data gave proof that the cell line tested was actually T24. Futher work is necessary to repeat the experiment with well characterised urothelial cells (Johnen et al., 2013) and re-evaluate the data presented here.

Aberrant MGMT promoter methylation leads to decreased defence abilities of the cell against genotoxicity. O^6-methylguanine-DNA methyltransferase (MGMT) transfers the alkyl groups from the O^6-position of guanine on itself. Since the protein is not regenerated after the reaction but degraded, this protein is not a true enzyme (Ekim et al., 2011, Pegg, 2011), and depletion of its functionality might lead to a dramatic accumulation of genetic damages. The experiments of the present work investigated the increase in MGMT promoter methylation in T24 cells following chronic treatment with MMA(III) for up to 80 weeks. Although T24 cells are tumour cells, the level of MGMT promoter methylation is low (approximately 4%) and comparable to normal urothelial cells (approximately 2%). In contrast, MGMT promoter methylation of hepatoma cells (HepG2) is more than 15 times higher (approximately 62%) (Johnen et al., 2013). As a result of the experiment conducted in the present study, no increase in MGMT could be observed (Fig. 43) after chronic MMA(III) exposure, leading to the conclusion that under the *in vitro* test conditions used, MMA(III) had no impact on the expression of MGMT. Further studies investigating arsenic-induced decrease of MGMT-functionality are deemed necessary, especialy in light of the genotoxic properties observed for arsenic compounds.

RAR ß is known as a nuclear receptor and a transcriptional factor, and together with its isoforms it was reported to be closely linked to cancer due to tumour suppressive

properties (Lei & Thé, 2003). Altered methylation patterns of RAR ß have also been detected in bladder cancer (Chan et al., 2002; Hoque et al., 2006; Negraes et al., 2008). However, no changes in the promoter methylation of RAR ß were observed in T24 cells during chronic low-dose exposure with MMA(III) up to more than 80 weeks as compared to concurrently non-treated control cells (Fig. 44). However, the base-line methylation patterns of RAR ß in untreated T24 control cells is already approximately 60%, but aggressive cancer cell lines like HeLa show methylation paterns of 98% (Johnen et al., 2013). Thus, a further influence of arsenic exposure on RAR ß methylation in existing urothelial tumour tissue seems in light of the present result unlikely.

Recently, the expression of C1QTNF6 was detected in hepatocellular cancer tissue, and interestingly, no C1QTNF6 protein was found in non-cancerous liver tissues. Moreover, its possible role in tumour angiogenesis in this cancer type was discussed (Takeuchi et al., 2011). Furthermore, endocrine actions, and anti-inflammatory functions of C1QTNF6 were reported (Wong et al., 2008; Kim et al., 2010). However, the pathologic role as well as the expression status of this protein in carcinogenis still remains unclear and needs further clarification and investigations. Therefore, the methylation pattern of the gene coding C1QTNF6 was analysed in the present work in order to identify a possible influence of C1QTNF6 protein expression on the progression of T24 cells during long-term exposure to MMA(III) (Fig. 46). For this purpose, DNA methylation was examined in T24 cells treated with 100 nM MMA (III) over a period of 84 weeks. The results revealed an increase of methylation in the treated cells when compared to the concurrently untreated control cells from week 36 onward. Starting from week 64, the increase became even more distinct, until an approx. 3.5 fold higher methylation status in week 84. Since hypermethylation leads to decreased protein expression, the biological consequence and the influence on the tumour progression remains unclear. It is unlikely, that the observed effect has a tumour promotive function, but a tumour protective function is even more questionable. Further research is urgently needed to clarify the role of C1QTNF6 protein and its expression in progression in arsenic-exposed T24 cells *in vitro* as a model for arsenic-induced bladder cancer.

The methylation status of CDH1, coding the cell adhesion protein E-cadherin, was reported not to be a suitable biomarker to differentiate between benign and malignant

tumours, as both show similar methylation patterns (Pu et al., 2006). Nevertheless, promoter hypermethylation of CDH1 was often found in epithelial tumours, resulting in the loss of inhibition of cell invasion (Yoshiura et al., 1995; Conacci-Sorrell et al., 2002). In the present work, promoter methylation of CDH1 was investigated in T24 cells after chronic treatment to 75 and 100 nM MMA(III). After 8 weeks of exposure the DNA methylation level of control cells was clearly higher than that of both treated cultures (Fig. 47 and Fig. 48), but intersecting at week 24 for the 75 nM culture and at week 36 for the 100 nM culture. From this time point on a time dependent decrease of CDH1 methylation in control cells and increase in treated cells was observed, leading to an even more distinct difference when comparing both control and treated cells. Due to the fact that such a clear difference in the promoter methylation was observed, a biological relevance may be assumed. Furthermore, this result is in compliance with the results received from the cellular migration and invasion assay, showing a distinct dose-dependent and treatment-related increase in cellular migration and invasion (Fig. 51). Hence, it can be assumed that promoter hypermethylation of CDH1 is one of the key events during MMA(III)-induced progression of T24 cells *in vitro*, leading to increased cellular motility and invasiveness. However, to prove this hypothesis, further experiments are neccessary, where hypermethylation of CDH1 is artificially suppresed. If progression would be hereby prohibited, srong evidence would be given that the hypothesis would be true.

Summarising all the data received for DNA methylation, it seems that this epigenetic process might only be affected to a minor degree by chronic arsenic exposure of T24 cells. However, further experiments analysing more genes known to be involved in carcinogenesis are needed to confirm this. Since the T24 cell line is tumourigen, the DNA methylation patterns have already distinctly changed, so that those prominent genes, which are generally analysed for the detection of tumourigenesis, have already undergone modifications. Typical examples in this study were LINE1 and RASSF1. During baseline establishment a change in the methylation status of T24 cells in contrast to “normal untransformed” urothelial cells was noted. T24 cells showed a decreased methylation of LINE1 (45% in T24 cells versus 75% in normal urothelial cells), whereas the following genes, which are usually unmethylated in primary urothelial cells, showed a distinct increase of DNA methylation: DAPK1 (20%), RAR ß (60%), CDH1 (22%), and RASSF1 (90%). It is assumed that due to

this altered background the changes in methylation resulting from arsenic treatment may have been superimposed. However, at study initiation, the cell line used was expected to be UROtsa, which is non-tumorigenic. But this cell line was generated by immortalisation with SV40, which also revealed changes to the genetic background and led to altered DNA methylation patterns too. Tab. 5 shows the DNA methylation of selected genes in different cell lines and normal urothelial cells from healthy patients, representing the difference in continous cell lines and primary cultures. The challenge for the further research will be to establish a continuous urothelial cell line exhibiting less altered epigenetic background changes. Primary cell cultures are not considered suitable for the purpose of such methylation studies, due to their short lifetime.

Tab. 5 DNA methylation in different cell lines

[1] UROtsa: urothelial cell line provided by A. Fabrarius, University of Heidelberg, Germany
[2] T24 cell line used in the present work (provided as UROtsa cell line by M. Styblo, University of North Carolina, USA)
[3] T24: transitional cell carcinoma of urinary bladder, available at ATCC (American Type Culture Collection)
[4] HepG2: hepatocellular carcinoma, available at ATCC
[5] HeLa S3: cervix carcinoma, available at DSMZ (German Collection of Microorganisms and Cell Cultures)
[6] Urotheal urine: mean of results obtained from four healthy persons (Johnen et al., 2013, modified)

	Gene promoter region (% methylation)					
Cell line	**LINE1**	**RAR ß**	**RASSF1**	**CDH1**	**C1QTNF6**	**MGMT**
UROtsa [1]	56	96	1	2	12	1
T24 [2]	46	64	91	23	9	4
T24 [3] (ACC 376)	47	50	93	83	18	1
HepG2 [4] (HB-8065)	54	5	89	7	31	62
HeLa S3 [5] (CCL-2.2)	69	98	3	97	7	2
Urothel urine [6]	69	2	2	3	18	2

5.4 The role of miRNAs in arsenic-induced carcinogenesis

At this point I want to express my gratitude to my colleagues and project partners Dr. D. G. Weber and Dr. O. Bryk from the Institute for Prevention and Occupational Medicine of the German Social Accident Insurance (Institute of the Ruhr-University Bochum, Germany) under the head of Dr. G. Johnen, who kindly performed the experiments of the miRNA analysis and provided the data for the present study.

Discussion of the role of miRNAs in arsenic-induced carcinogenesis

In tumour tissue the regulation via miRNAs is of great relevance. Among classically known cancer genes, miRNAs are known as molecular modulators, and were described as essential components of fundamental signal transduction of carcinogenesis (Dalmay & Edwards, 2006; Sotiropoulou et al., 2009). Comparable to protein-coding genes miRNAs can also be downregulated by DNA hypermethylation in the promoter regions of cancer cells (Zimbardi et al., 2012). Many miRNAs possess tumour suppressive functionality and can depress proliferation. Altered miRNA profiles in bladder cancer tissues were recently reported (Catto et al., 2009; Dyrskjot et al., 2009; Ichimi et al., 2009; Lin et al., 2009; Veerla et al., 2009; Hanke et al., 2010). Nevertheless, only little information is available on the effect of arsenic treatment on miRNA profiles, especially in bladder cancer. To gain more information on the regulatory impact of miRNAs in arsenic-induced bladder cancer, an expression profile of 33 miRNAs associated with cancer was analysed. T24 cells derived from the chronic treatment with 100 nM MMA(III) and concurrently untreated control cells were harvested every 4 weeks starting from week 10 up to week 56 of exposure. Although the expression profile revealed various fluctuations of the analysed miRNAs throughout the study period, there were few miRNAs showing distinct trends in their profiles (Tab. 4). These were namely miR-139-5p, miR-146a, miR-149, miR-200a, and miR-429, which were only recently associated with bladder cancer in particular (Catto et al., 2009; Dyrskjot et al., 2009; Ichimi et al., 2009; Lin et al., 2009; Veerla et al., 2009). In the present study miR-139-5p was significantly decreased at six time points from week 32 onwards, whereas 2 and 4 time points were hereby in direct succession. MiR-139-5p was recently reported to be downregulated in various kinds of cancer diseases, including basal cell carcinoma (Sand et al., 2012), ovarian cancer (Miles et al., 2012), and clear cell renal cell carcinoma (Wu et al., 2012). Especially in the latter carcinoma, miR-139-5p was associated with metastasis and poor prognosis. Additionally, miR139 was detected to be decreased in bladder tumours (Ichimi et al., 2009), possibly targeting CXCR4 and resulting in metastasis. CXCR4 was described to be increased in invasive bladder tumours (Dalmay & Edwards 2006; Retz et al. 2005). In the present work, miR-200a was also found to be significantly decreased in 100 nM MMA(III)-treated T24 cells as compared to concurrently untreated control cells at week 19, 28, and continuously

from week 36 onward. MiR-200-miRNAs inhibit the expression of the proteins Zeb1 and Zeb2, which suppress the transcription of CDH1. Decreased levels of miR-200-miRNAs were correlated with progression in breast cancer, and further with the poor prognosis of ovarian carcinoma (Gregory et al., 2008; Hu et al., 2009). The findings match with the results from the present work, since enhanced migration and invasion of treated cells were also observed, indicating an even more malignant phenotype with properties required for metastasis (Fig. 51). As in case of miR-200a this would mean that the synthesis of the E-cadherin protein could have been depressed via two independent routes: first, as a secondary effect of the decrease of members of the miR-200-miRNA family, and second, as a result of CDH1 hypermethylation as described earlier (Fig. 47 and Fig. 48).

MiR-146a was detected to play a role in suppression of metastasis in breast cancer (Hurst et al., 2009), among others, by decreasing protein levels of MMP9, which is important for the invasion of tumour cells (Bhaumik et al., 2008). ROCK1 was identified to be a target of miR-146a in prostate carcinoma cell lines, as well as CXCR4 in leukemia cells (Labbaye et al., 2008; Lin et al., 2008). In this study miR-146a expression was significantly and continuously decreased from week 32 of exposure until the end of the study, giving further evidence that alteration of miRNAs expression profiles may contribute to tumour progression in MMA(III)-treated T24 cells. In contrast, miR-149 was observed to be increased during the study at weeks 10, 24, 32, and 40-52. Zhaohui et al. (2011) reported the miR-149 mediated promotion of proliferation of nasopharyngeal carcinoma cell lines, as well as an increase in their mobility and invasion. Furthermore, miR-149 was reported to downregulate the expression of E-cadherin. The authors conclude that miR-149 might be involved in the invasion and metastasis continuously through the regulation of the epithelial-mesenchymal transition (EMT). Regulation of EMT through miRNAs was further discussed for the miR-200-miRNAs (miR-200a, -200b, -200c, -141, -429) in combination with miR-205. As described earlier, significant changes in the expression of miR-200a were observed. Furthermore, miR-205 was clearly increased at 4 time points throughout the study (week 19 36, 52, and 56), even though fluctuations were observed; in addition, miR-429 expression was also altered throughout the study period. After first fluctuations (decrease at weeks 10 and 28 and increase at weeks 15 and 24), a continuous decrease was detected from week 36

onward, suggesting the hypothesis of a compensatory mechanism at the beginning, which finally failed, leading to the further development of a malignant phenotype. Since the development of the expression profile of miR-429 led to the hypothesis of a compensation process during malignancy, the analysis was repeated and the expression profile was not only measured for 100 nM MMA(III) exposure, but also for 50 and 75 nM MMA(III), as well as 50 nM As(III) (Fig. 41). In all treatment groups a strong increase of miR-429 was observed after 15 weeks of exposure, followed by a strong and more or less steady decrease far below the control values and the values obtained in the treated groups at test beginning. The increase was the strongest at 50 nM MMA(III) and moderate at 75 nM MMA(III), whereas at 100 nM MMA(III) the increase was no more statistically significant. In the case of As(III), the weakest increase of miR-429 was observed. The analogue trend, the dose dependency, and the fact that both MMA(III) and As(III) were affected from the increase, enhance the hypothesis that a compensatory mechanism had occurred, but finally failed.

Summarising all the data derived from the miRNA analysis, some evidence is given that altered miRNA profiles play a pivotal role in arsenic-induced progression of T24 cells *in vitro*. Nevertheless, care has to be taken when the relevance of altered miRNA profiles is interpreted in carcinogenesis, as their expression patterns are highly specific for cell-type and cellular differentiation status. This is further highlighted by the findings of Johnen et al. (2013), who compared the miRNA expression in different cell lines (Fig. 53).

Altered expression patterns are not conclusively the reason for malignant transformation, but a consequence of genetic and epigenetic changes that result in the loss of the normal cell identity accompanied by malignant transformation (Kent & Mendell, 2006). Further *in vivo* experiments are necessary to confirm the results of the present work, and to clarify the regulatory impact of miRNA expression in arsenic-induced bladder cancer in humans.

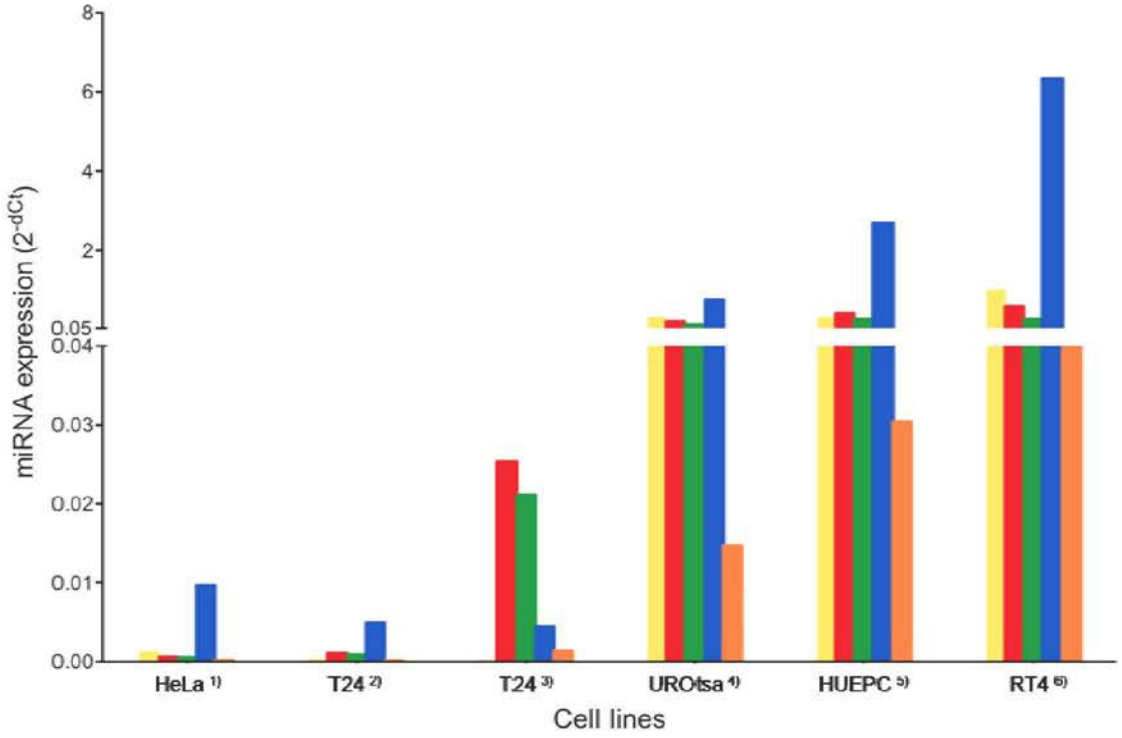


Fig. 53 Comparison of miRNA expression in different cell lines.

Normalized levels of miR-141 (yellow), miR-200a (red), miR-200b (green), miR-200c (blue), and miR-429 (orange) were determined in HeLa [1)], T24 [2)], T24 [3)], UROtsa [4)], RT4 [6)], and HUEPC [5)]. Expression levels were determined by Real-Time 22 PCR. RNU44 and RNA48 were used for normalization. The y axis was adapted to better accommodate the genes with low expression levels. (Johnen et al., 2013, modified)

[1)] HeLa S3 (DSMZ); [2)] T24 (ATCC); [3)] T24 cell line used in the present work (provided as UROtsa) (Styblo, University of North Carolina, USA); [4)] UROtsa (Fabrarius, University of Heidelberg, Germany); [5)] HUEPC (Provitro GmbH); [6)] RT4 (well differentiated transitional papillary tumour of urinary bladder) (ATCC)

5.5 Arsenic-induced COX-2 protein activation

The COX-2 protein is known to exhibit pro-carcinogenic properties (Vane et al., 1998). It was reported to suppress apoptosis, promotes proliferation, and is involved in many processes during the development of various cancer diseases including bladder cancer, and moreover, malignant transformation of human urothelial cells (UROtsa) as a consequence of chronic low-dose treatment (Eblin et al., 2007). COX-2 protein was described to be an essential factor for the survival of human and mammalian cells under stress conditions, as it protects the cells from stress-induced apoptosis, for example caused by DNA damaging agents (Chai et al., 2007). The induction, expression, and activity of COX-2 was reported to be one essential step in the development of colon cancer due to the decrease of apoptosis and the survival of the epithelial cells beyond normal lifetimes, allowing the development of a malignant

phenotype. Therefore, COX-2 activity is correlated with the progression of pre-cancerous epithelial cells to fully malignant phenotypes (Vane et al., 1998). In general, there is no COX-2 activity in the tissues, but a single stimulus enhances the protein level for only a few hours (Vane et al., 1998). In the present study the occurrence of COX-2 protein in T24 cells was investigated after arsenic exposure in comparison to concurrently untreated control cells. Treatment with 75 and 100 nM MMA(III) for up to 1 week resulted in first a distinct decrease in intracellular COX-2 protein after 1 h, which normalised to control values again within 24 h (Fig. 38). Already after 1 week of exposure to both 75 and 100 nM MMA(III), elevated COX-2 protein levels as compared to untreated controls were observed. The effects detected showed a dose-response, with 100 nM treatment resulting in a slightly stronger decrease within 1 h and a slightly stronger increase after 1 week of exposure as compared to 75 nM treatment. Chronic exposure of T24 cells to MMA(III) revealed more or less continuously elevated COX-2 protein levels in treated cells as compared to concurrent control cells (Fig. 39). Fluctuations were observed for both exposure concentrations, whereas, in contrast to the short-term treatment, the increase of COX-2 protein was stronger in 75 nM-treated cells than in those treated with 100 nM. While 100 nM MMA(III)-treated cells showed lower COX-2 protein content than the control cells during weeks 25 - 33 of exposure, and the values were comparable to those of the control cells again from week 60 to week 65, treatment with 75 nM MMA(III) never resulted in values below those of the control cells and only at week 28, a COX-2 protein level comparable to that of the control was noticed. The fluctuations suggest that there could be a compensatory processe occurring to prevent the development of cell malignancy. To investigate the regulation of the COX-2 protein expression, the respective mRNA level was also analysed during the chronic low-dose exposure to MMA(III) (Fig. 40). The mRNA levels detected in 100 nM-treated and untreated cells ran inversely proportional. Fluctuations such as those seen from the protein content were not observed for the mRNA level from week 32 onward. While COX-2 protein expression in exposed cells was elevated as compared to the control between week 40 and week 56, the mRNA level of 100 nM-treated cells was decreased at the same time. Hence, it can be concluded, that the regulation of the COX-2 protein content in the cells is not induced by the existing amount of the respective mRNA. Interestingly, the trend of the COX-2 mRNA

observed in 100 nM MMA(III)-treated cells matches with the trend of the miR-26a and miR-26b (Fig. 54). This result is in compliance with the findings of Ji et al. (2012), showing that the expression levels of COX-2 mRNA and miR-26b were inversively correlated in carcinoma cells of nasopharyngeal epithelia (CNE cells).

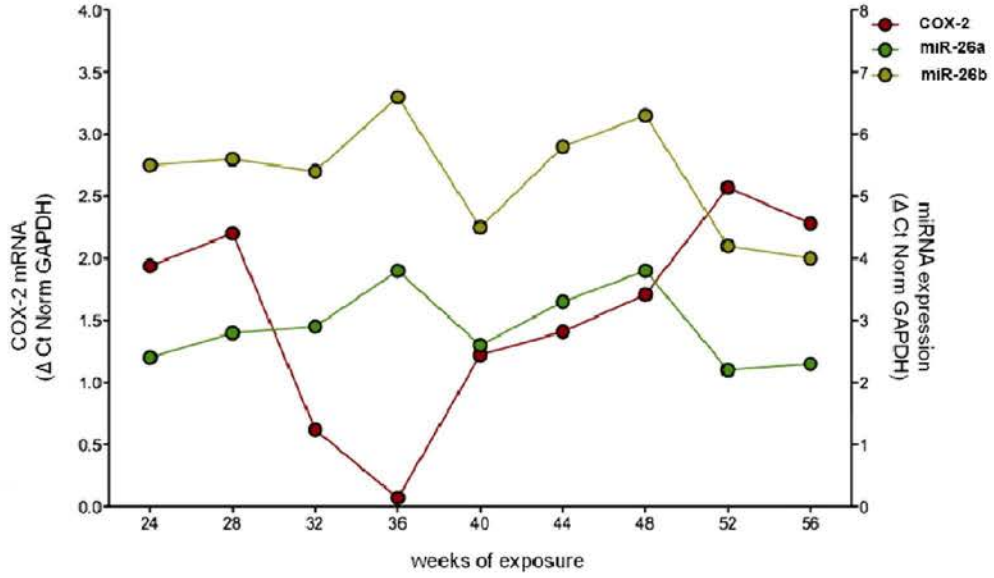


Fig. 54 COX-2 mRNA (normalised) and miR-26a- and miR-26b- expression (normalised) in UROtsa cells after exposure to 100 nM MMA(III) for 24 - 56 weeks

The authors suggest that COX-2 mRNA contains a binding site for miR-26b, which is suspected to regulate the COX-2 protein expression by directly interacting with the mRNA. The authors further suggested mRNA cleavage as a possible mechanism. This assumption could not be confirmed in the present study. While direct interaction between miR-26a and miR-26b with the COX-2 mRNA can be assumed (Fig. 54), it is unlikely that this alone leads to direct regulation of the COX-2 protein synthesis, since the mRNA level and the protein level could not be directly correlated with each other (Fig. 39 and Fig. 40). It might be possible that further events occur during the chronic treatment with MMA(III), which influence the COX-2 protein expression by interfering with the regulatory effect of miR-26a and miR-26b. Moreover, it is generally known that several backup systems exist to prevent aberrant cell proliferation and malignant transformation. It is likely that exposure to MMA(III) induces deregulatory and, in addition, compensatory processes, finally leading to an imbalance, which would also explain the strong fluctuations observed for COX-2 mRNA and protein level, as well as for the miRNA levels. It is possible that with longer exposure times this imbalance

would increase, finally leading to a failure of the compensatory system and maybe revealing a clear inverse connection between the miRNAs, the COX-2 mRNA, and the respective protein level. Nevertheless, further experiments with longer exposure durations are necessary to confirm this hypothesis.

5.6 Arsenic-induced tumour progression

The processes described above are by far not expected to be the only ones ongoing in urothelial cells during arsenic-induced malignant transformation. But these processes are believed to be part of the key events occurring during tumour development and progression. To prove direct correlation of those mechanisms with the development of tumour progression of T24 cells as a result of arsenic exposure, further experiments were carried out to analyse malignant phenotypical alterations. T24 cells derived from the chronic low-dose exposure to MMA(III) were included into several tests, investigating effects such as altered morphology, growth inhibition, anchorage-independent growth, motility, and invasiveness.

At first, the *in vitro* Mammalian Cell Transformation Test was carried out. This test analyses the possible loss of contact inhibition during tumour progression and is mentioned in the EU method B.21 for testing mutagenicity of chemicals. T24 cells derived from the long-term exposure to 50, 75, and 100 nM MMA(III) were seeded to confluence. The non-tumourigenic variant of T24 cells normally grow in a monolayer, and hence, if no tumour progression had occurred yet, the cells would have stopped proliferating (Gildea et al., 2000). This behaviour physiologically preserves the normal organ shapes in organisms. In this study, untreated control cells showed the more or less physiologic behaviour, supporting the assumption that the T24 cell line contaminating the UROtsa stock used in our laboratory is the non-tumourigenic variant. However, chronic exposure to MMA(III) led to a loss of this preventive contact inhibition and revealed cells growing in three-dimensional layers (Fig. 49). Within two weeks of incubation the cells formed colonies with a diameter of more than 200 µm. Moreover, the treated cultures included big and misshapen cells. The observations were strongest for the 75 nM-treated culture, but also 50 nM-exposed cells exhibited altered morphology and colonial growth. At 100 nM MMA(III) treatment, the cells built up only few and barely perceptible colonies, but misshapen

cells were still recognisable. The fact that untreated control cells of the same passage did not exhibit such an altered phenotype, clearly indicates that the effects observed are treatment-related and suggest that MMA(III) treatment induces tumour progression. Altered morphology of urothelial cells as a result of long-term low-dose treatment with arsenic was also reported for the untransformed cell line UROtsa (Bredtfeld et al., 2006); and the detected loss of contact inhibition matches with the findings that the expression of the cell surface protein E-cadherin was affected by arsenic treatment (induced by CDH1 promoter hypermethylation (Fig. 47 and Fig. 48) and altered expression profiles of the miR-200 family miRNAs (Tab. 4 and Fig. 41). E-cadherin is normally connected with cell surface receptors such as the EGFR, enhancing cell-cell attachments and, moreover, disabling growth factors to bind to their respective receptors. This connection is carried out by the protein Merlin, which is a gene product of NF2 (Okada et al., 2005, Curto et al., 2007, Hanahan & Weinberg, 2001). Further experiments analysing e.g. promoter hypermethylation of NF2 or the expression profiles of Merlin might give further insights into the mechanism of arsenic-induced tumour progression, in particular, into the mechanism underlying the loss of contact inhibition.

The results observed in the *in vitro* Mammalian Cell Transformation Test are similar to those observed in the Colony Formation Assay. This assay aims to detect anchorage-independent growth by bedding the cells into soft agar. Adherent growing cells, such as the non-tumourigenic variant of T24 cells, suffer apoptosis, if they fail to attach to a surface (Gildea et al., 2000). This effect was shown for the untreated control cells, which failed to grow under these conditions (Fig. 50 D). In contrast, T24 cells treated with MMA(III) for 93 weeks clearly exhibited distinct growth abilities in soft agar, confirming the loss of anchorage dependent growth (Fig. 50 A-C). This phenotype was also noted for the tumourigenic T24T cell line (Gildea et al., 2000). It can therefore be assumed that under the conditions of this study, MMA(III)-treatment caused tumour progression in human bladder cells *in vitro*.

The observation of anchorage independent growth might indicate that those cells *in vivo* would not underlie elimination by apoptosis, as soon as they are released from their respective tumour tissue. As a consequence, the cells should now be able to circulate in the organism once they have entered the blood stream. Hence, the ability of anchorage-independent growth is one essential step in the development of

metastasis. Moreover, only a few of the MMA(III)-treated T24 cells surviving in soft agar in this study grew separately; tumour-like colony formation was the predominant observation. This effect was observed over all three MMA(III) concentrations tested, but treatment with 75 nM MMA(III) exhibited the biggest and most dense colonies. This finding matches with the fact that COX-2 activation was stronger in 75 nM-treated cells than those exposed to 100 nM MMA(III), since elevated COX-2 protein expression was reported to be important for sustaining anchorage-independent growth (Eblin et al., 2009). The results not only suggest that the MMA(III) treated cells underwent tumour progression to develop the ability to form primary tumours, but might also gain properties to form metastases. Bredtfeld et al. (2006) observed anchorage-independent growth of UROtsa cells exposed to 50 nM MMA(III) for 24 and 52 weeks. Moreover, xenograft implantation of these cells into SCID mice exhibited the formation of moderately differentiated cell carcinomas (Bredfeldt et al., 2006). Since UROtsa cells were described as generally non-tumourigenic (Petzold et al., 1995; Sens et al., 2004; Bredtfeld et al, 2006), the results clearly prove arsenic-induced malignant transformation. The tumourigenic T24T cell line was also shown to form tumours when injected into SCID mice (Gildea et al., 2000). Consequently, implantation of T24 cells derived from the long-term exposure to 50, 75, and 100 nM MMA(III) of this study into SCID mice should lead to similar results, but possibly giving further information on dose dependency, which was not investigated by Bredfeldt et al. (2006). It would be interesting to see, if MMA(III) treatment also reveals strongest tumourigenic effect at 75 nM like in the Colony Formation Assay in soft agar.

Metastasis is a later stage in carcinogenesis that in most cases is the immediate cause of cancer-related death. In order to metastasise tumour cells have to increase their motility and must be able to invade through tissues and the walls of blood and lymphatic vessels, which is essential for the extra- and intravasation process (Fidler, 2003, Fig. 10). In this study the motility and the invasive potential of T24 cells derived from the chronic low-dose exposure were investigated using the xCELLigence system. The cells were seeded into devices consisting of two chambers separated by a microporous membrane. Cells migrating through this membrane were detected using impedance measurement. Values revealed from concurrently untreated control cells were compared to those of cells treated with MMA(III). The results show that

untreated T24 cells exhibit a certain migration potential, which corresponds to the observations made by Gildea et al. (2000). However, motility of cells exposed to 75 and 100 nM MMA(III) for more than 90 weeks was distinctly increased. Only the 50 nM-treated cells exhibited decreased motility as compared to the control (Fig. 51 A). In further experiments the microporous membranes of the devices were coated with collagen on both sides as a simulation of extracellular matrix (Du et al., 2009). Those cells, which gained proteolytic properties and the ability to pass the membrane and penetrate its coating, are supposed to be able to invade through tissues and the walls of blood and lymphatic vessels, and hence, are strongly malignant exhibiting metastatic potential. Untreated control cells of the T24 cell line exhibited invasive properties, which was also reported by Gildea et al. (2000), but after 92 weeks of exposure cells treated with MMA(III) showed a strong increase in invasion as compared to the untreated control cells (Fig. 51 B). While invasion of 50 and 75 nM-treated cells increased only to a slight but still distinct degree, 100 nM-treated cells invaded within the 20 h experiment duration 2.5 imes more than the control cells and approx. 2 times more than the 50 and 75 nM-treated cells. Moreover, invasion of 100 nM-treated cells was by far quicker than that of the control cells and the lower treatment doses, exhibiting a linearity and therefore a maximum velocity between 6 and 10 h experiment duration, finally leading to an experiment-based saturated state. In contrast, the course of invasion of 50 and 75 nM-exposed cells was parallel to that of the untreated control cells, and none of these samples reached the saturated state. These findings support a dose-response relationship and suggest that the malignant effect increases with the exposure concentration. Nevertheless, when comparing all endpoints addressed in this study, the dose-dependency is inconclusive. Among this, care must be taken when endpoints addressing malignancy are evaluated in T24 cells, since this cell line is already a cancer cell line. Nevertheless, the tumourigenic T24T cell line was reported not to exhibit increased invasive properties as compared to the non-tumourigenic variant T24. As an increase was observed in T24 cells after chronic MMA(III) treatment, it can be assumed that the phenotype resulting from arsenic exposure might be even more malignant than that presented by the T24T cell line. A further uncertainity is given by the fact that the experiment with chronic MMA(III) exposure was carried out only once owed to the huge experimental effort. Thus, for further certainity, the

experiments described here should be repeated and the results should be evaluated again. Moreover, experiments should additionally be carried out with the normal untransformed UROtsa cell line. Since the methods applied analysing phenotypical alterations after chronic arsenic exposure revealed positive results, it can be expected that similar (or even worse) effects will be observed when testing UROtsa. Unfortunately, there is too little information given in the scientific literature, supporting or negating the hypothesis of dose-dependent malignant transformation of arsenic-treated T24 or UROtsa cells. Nevertheless, few data is available for 50 nM MMA(III) and 1 µM As(III) in comparison to 1 µM Cd(II). Jensen et al. (2009) summarised the phenotypic alterations of UROtsa cells observed during development of malignancy after chronic low-dose exposure to MMA(III) (Tab. 6).

Tab. 6 Summary of arsenic exposure conditions and selected effects on UROtsa cells as a result of various recent studies

Cell line name, the treatment metal, concentration (exposure), and duration of treatment for each cell line are shown. In addition, the phenotypic properties of each cell line including increased growth rate relative to UROtsa (hyperproliferation), anchorage-independent growth (AIG), and ability of each cell line to form tumours when injected subcutaneously into immunocompromised mice are described. The reference cites previous publications describing part or all of the information presented for a given cell line. (Jensen et al., 2009, modified) (NA = not applicable; ND = not determined)

Cell Line	Treatment	Exposure	Duration	Hyperproliferation	AIG	Tumors in mice
UROtsa	None	None	None	NA	No	No
URO-MSC12	MMA (III)	50 nM	12 weeks	Yes	No	No
URO-MSC24	MMA (III)	50 nM	24 weeks	Yes	Yes	No
URO-MSC36	MMA (III)	50 nM	36 weeks	Yes	Yes	ND
URO-MSC52	MMA (III)	50 nM	52 weeks	Yes	Yes	Yes
URO-MSC24 + 3mo	MMA (III)	50 nM	24 weeks	ND	Yes	ND
URO-MSC24 + 6mo	MMA (III)	50 nM	24 weeks	ND	Yes	ND
URO-MSC52 + 3mo	MMA (III)	50 nM	52 weeks	ND	Yes	Yes
URO-MSC52 + 6mo	MMA (III)	50 nM	52 weeks	ND	Yes	Yes
URO-ASSC	As (III)	1 µM	52 weeks	Yes	Yes	Yes
URO-CDSC	Cd (II)	1 µM	52 weeks	Yes	Yes	Yes

5.7 Conclusion

In conclusion, further studies are necessary to gain more information on the impact of the exposure concentrations. Since arsenic is not only carcinogenic at low doses, it also exhibits cytotoxic effects at higher concentrations, leading to strong systemic toxicity *in vivo*. It is therefore likely, that at one point the exposure concentration exceeds the "tolerance level", finally suppressing carcinogenicity due to the

predominance of acute toxicity. More studies, both *in vitro* and *in vivo*, have to investigate a possible cut-off value for carcinogenesis. In addition, more epidemiologic information is needed about human exposure and the concurrent health hazard. Nevertheless, elimination of human arsenic contamination should be the ambitious aim by e.g., applying clean water technologies, minimising occupational exposure, and avoiding contaminated food.

Fig. 55 summarises the molecular mechanisms of MMA(III)-induced toxicity and malignancy in T24 cells *in vitro* after chronic low-dose exposure. This extended model proposes the fast cellular uptake and subsequent conjugation of MMA(III) to proteins and other cellular structures in addition to the immediate occurrence of genotoxicity. It can be hypothesised that, as a "first-response" to such a cellular stress, cellular autophagy is activated, preventing cell death and, as a side effect, causing a kind of "cellular cycling" of MMA(III). In addition, the affection of cellular structures by MMA(III) leads to epigenetic alterations, which can be manifested and might become inheritable because of the prevented cell death. As a consequence, malignant transformation and progression can occur, leading to pre-neoplastic cells. This finally might reveal the development of cancer *in vivo* (Hippler et al., 2011; Zdrenka et al., 2012).

Because this highly hypothetic model is based on few endpoints addressed in *in vitro* research using urothelial carcinoma cells, further experimens should be conducted to verify the results observed. It is likely that few effects such as increased DNA methylation of RASSF1 resulting from chronic exposure to MMA(III) are invisible, because a maximum alteration is already present in the cancer cell line tested. However, in case of the positive tests, it can be expected that the effects observed would be even worse with normal, untransformed cells, since arsenic exposure increased the malignant potential of an already malignant cell line. However, care must be taken when interpreting the results obtained from the present study: owing the huge experimental effort necessary for chronic treatment of cell cultures and continous sampling of these during that exposure time, the experiment was conducted with one culture per test concentration only. Thus, an experimental design should be developed, in which more cultures of the same treatment group can be run in parallel.

Among good quality data obtained from *in vitro* tests, there is still an urgent need for further *in vivo* studies. While *in vitro* assays give important data for the investigation of molecular mechanisms, there is lack of information concerning the defence of a whole organism against cancer, including among others, toxicokinetic and toxicodynamic of the carcinogen, immune response, and tumour growth restriction because of limited blood supply. Only *in vivo* studies have the power to correlate the mechanism observed on single cell basis *in vitro* with a realistic carcinogenic potential. Nevertheless, *in vitro* research enables researchers to investigate specific targets in animal experiments without being lost in the complexity of a whole organism. Determining *in vitro* tests as a first step enables researchers to define a concrete question for the subsequent *in vivo* experiment, and hence, decreasing animal numbers in terms of animal welfare.

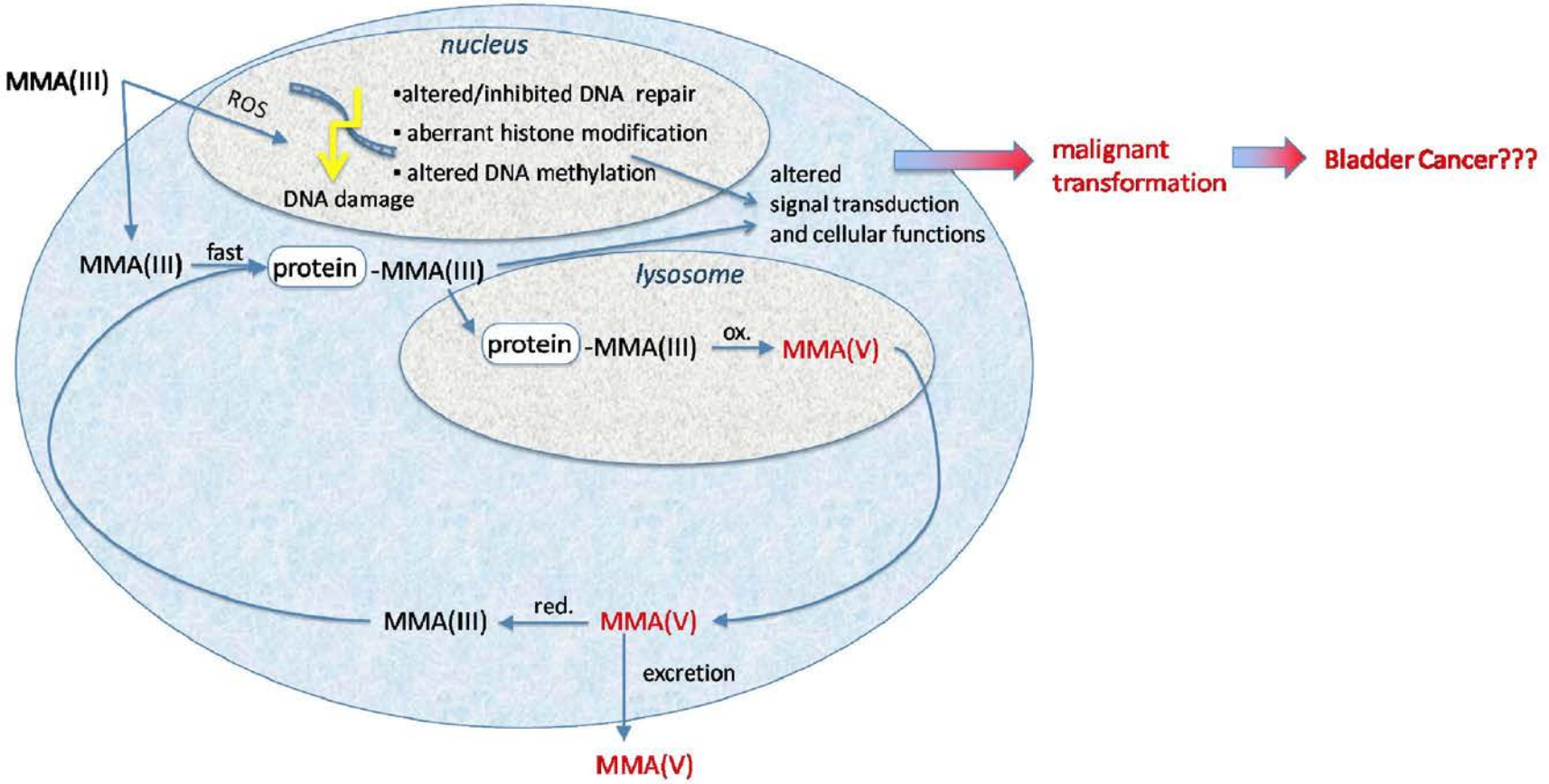


Fig. 55 Proposed molecular mechanisms of MMA(III)-induced toxicity and malignancy in UROtsa cells after chronic low-dose exposure

(Zdrenka et al., 2012)

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7 Annex

7.1 Instruments, chemicals and reagents

Laboratory equipment used in this study

➢ Water-Jacked Incubator	Forma Scientific (Marietta, USA)
➢ Microscope Labovert FS	Leica Microsystems GmbH (Wetzlar, Germany)
➢ Digital camera for the Microscope Labovert FS	Leica Microsystems GmbH (Wetzlar, Germany)
➢ Fluorescence microscope DMLS	Leica Microsystems GmbH (Wetzlar, Germany)
➢ Digital camera for the fluorescence microscope	Leica Microsystems GmbH (Wetzlar, Germany)
➢ Centrifuge MiniSpin plus	Eppendorf (Hamburg, Germany)
➢ Centrifuge Microfuge 22R Cent	Beckmann Coulter (Krefeld, Germany)
➢ HPLC 1100	Agilent Technologies (Waldbronn; Germany)
➢ HPLC Coloumn Luna, 3µ C18(2), 150 x 4,6 mm, max. pressure 400 bar	Phenomenex (Aschaffenburg, Germany)
➢ SecurityGuard Cartride Kit Cartridge: C18; 4 x 3.0 mm	Phenomenex (Aschaffenburg, Germany)
➢ ICP/MS 7500a	Agilent Technologies (Waldbronn; Germany)
➢ Precellys 24 Tissue Homogenisator	PEQLAB Biotechnologie (Erlangen, Germany)
➢ QIAcube	QIAGEN (Hilden, Germany)
➢ NanoDrop ND-2000	PEQLAB Biotechnologie (Erlangen, Germany)
➢ Sample-Prep Workstation	QIAGEN (Hilden, Germany)
➢ Pyrosequencer PSQ 96MA	QIAGEN (Hilden, Germany)
➢ GENios Plate Reader	Tecan (Crailsheim, Germany)
➢ xCELLigence DP System	Roche Applied Science (Mannheim, Germany)

Materials used in this study

➢ Cell culture flasks	TPP (Trasadingen, Switzerland)
➢ Cell Culture Multiwell Plate, 24 well	Greiner Bio-One (Frickenhausen, Germany)
➢ Microplate, 96 well	Greiner Bio-One (Frickenhausen, Germany)
➢ Cellscraper	Greiner Bio-One (Frickenhausen, Germany)
➢ Pipetus	Hirschmann Laborgeräte GmbH & Co. KG (Eberstadt, Germany)
➢ Serological Pipettes	Greiner Bio-One (Frickenhausen, Germany)
➢ Eppendorf Research Pipettes	Eppendorf AG (Hamburg, Germany)
➢ Eppendorf Research Multichannel-Pipettes	Eppendorf AG (Hamburg, Germany)
➢ Universal pipette tips	Greiner Bio-One (Frickenhausen, Germany)
➢ Reaction tubes	Greiner Bio-One (Frickenhausen, Germany)
➢ PS tubes screw cap	Greiner Bio-One (Frickenhausen, Germany)
➢ Precellys Homogenisation kit (0,5 ml tubes, ceramic 1,4 mm)	PEQLAB Biotechnologie (Erlangen, Germany)
➢ Injection filters 4 mm (0,2 / 0,45 µm)	Phenomenex (Aschaffenburg, Germany)
➢ GelBond Film	Lonza Group Ltd (Basel, Switzerland)
➢ Chamber Slides	Nunc GmbH &. Co. KG (Langenselbold, Germany)
➢ Immuno 96 MicroWell Solid PlatesMaxiSorp	Nunc GmbH &. Co. KG (Langenselbold, Germany)
➢ CIM-Plates for xCELLigence DP System	Roche Applied Science (Mannheim, Germany)

Cell lines and primary cells used in this study

➢ UROtsa cells (actually T24 cells)	Prof. M. Styblo (University of North Dakota, USA)
➢ HepG2 cells	ATCC (Manassas, VA, USA)
➢ HUEPC	Provitro GmbH (Berlin, Germany)

Chemicals and reagents used in this study

As(III), sodium arsenite, 98%	Fluka (Seelze, Germany)
As(V), disodium arsenate heptahydrate, 98%	Sigma-Aldrich (Taufkirchen, Germany)
MMA(III), monomethylarsonic iodide, 98%	Argus Chemicals (Vernio, Italy)
MMA(V), disodium monomethylarsonic acid, 98%	Tri-Chemical Laboratories Inc (Yamanashi, Japan)
DMA(III) dimethylarsinc iodide, >99%	Synthesis according to Styblo et al. (1997)
DMA(V) cacodylic acid, 99.5%	Strem (Kehl, Gemany)
MEM (Earle's Minimal Essential Medium)	cc-Pro (Oberdorla, Germany)
serum-free urothelial cell growth medium	Provitro GmbH (Berlin, Germany)
FBS (fetal bovine serum)	GIBCO Invitrogen (Darmstadt, Germany)
L-Glutamin	Sigma (Oberhaching, Germany)
NEAA (not essential amino acids)	cc-Pro (Oberdorla, Germany)
Sodium pyruvate	cc-Pro (Oberdorla, Germany)
Gentamycin	cc-Pro (Oberdorla, Germany)
Amphotericin B	cc-Pro (Oberdorla, Germany)
PBS (phosphate buffered saline)	Gibco Invitrogen (Darmstadt , Germany)
Trypsin	cc-Pro (Oberdorla, Germany)
Trypsin / EDTA	cc-Pro (Oberdorla, Germany)
Ampuwa (pure water)	Fresenius Kabi GmbH (Bad Homburg, Germany)
DMPS (2,3-bis(sulfanyl)propan-1-sulfonic acid)	Alfa Aesar (Karlsruhe, Germany)
Proteinase K	Sigma-Aldrich (Steinheim, Germany)
H_2O_2, 30%	Merck (Darmstadt, Germany)
Methanol, 99%	Merck (Darmstadt, Germany)
Tetrabutylammoniumhydroxide, 99.9%	Sigma-Aldrich (Steinheim, Germany)
Malonic acid, > 99%	Merck-Schuchhardt (Hohenbrunn, Germany)
Acetonitrile, > 99 %	Merck-Schuchhardt (Hohenbrunn, Germany)
THF (Tetrahydrofurane)	Fisher Scientific (Schwerte, Germany)
TFA (Trifluor acetic acid)	Fisher Scientific (Schwerte, Germany)
Pure water derived from the pure water device PURELAB ultra 18,2 MO * cm	Elga Labwater (Celle, Germany)
ENU (N-ethyl-nitroso urea)	Sigma-Aldrich (Steinheim, Germany)
LMP (low melting point) Agarose	Invitrogen (Darmstadt, Germany)
Trizma HCl (2-Amino-2-(hydroxymethyl)-1,3-propanediol)	Sigma-Aldrich (Steinheim, Germany)
NaOH (sodium hydroxide)	Sigma-Aldrich (Steinheim, Germany)
EDTA (Ethylenediaminetetraacetic acid)	Sigma-Aldrich (Steinheim, Germany)
NaCl (sodium chloride)	Sigma-Aldrich (Steinheim, Germany)
N-Lauroylsarcosine sodium salt	Sigma-Aldrich (Steinheim, Germany)
DMSO (dimethyl sulfoxide)	Sigma-Aldrich (Steinheim, Germany)
Triton-X	Merck (Darmstadt, Germany)
Glacial acetic acid	Sigma-Aldrich (Steinheim, Germany)
HCl (hydrochloric acid), 37%	Merck (Darmstadt, Germany)
SYBR Green	Invitrogen (Darmstadt, Germany)
Buffer ATL	QIAGEN (Hilden, Germany)
Proteinase K	QIAGEN (Hilden, Germany)
QIAamp DNA Mini QIAcube Kit	QIAGEN (Hilden, Germany)
Epitect Bisulfite Kit	QIAGEN (Hilden, Germany)
AmpliTaq Gold	Life TechnologiesCorporation (Carlsbad, CA, USA)
RNAlater	Life TechnologiesCorporation (Carlsbad, CA, USA)
RNeasy Plus Mini Kit	QIAGEN (Hilden, Germany)
TaqMan MicroRNA Reverse Transcription Kit	Life TechnologiesCorporation (Carlsbad, CA, USA)
TaqMan MicroRNA Assay	Life TechnologiesCorporation (Carlsbad, CA, USA)
RNase-free, sterile-filtered water	Life TechnologiesCorporation (Carlsbad, CA, USA)
CytoBuster	Merck (Darmstadt, Germany)
DC Protein Assay Reagents Package	BioRad Laboratories GmbH (Munich, Germany)
Protein Standard II, bovine serum albumin	BioRad (Munich, Germany)
COX-2 ELISA kit	Merck (Darmstadt, Germany)
Collagen	SERVA Electrophoresis GmbH (Heidelberg, Germany)

Solutios prepared in this study(Comet Assay)

➢ Tris solution	1 M Trizma HCl in Ampuwa
➢ NaOH solution	2 M NaOH in Ampuwa
➢ EDTA solution	0.5 M and 1 M EDTA in Ampuwa → adjust to pH 8 by using NaOH solution
➢ Lysis solution I	- 10 mL Tris solution (1 M) - 146.1 g NaCl - 100 mL EDTA (1 M) - 10 g N-Laurylsarcosine sodium salt → fill up to 1000 ml with Ampuwa and heat up to 100 °C
➢ Lysis solution II	- 100 mL DMSO - 10 mL Triton-X → storage under protection from light
➢ Neutralisation solution	- 200 mL Tris solution - 300 mL Ampuwa → adjust to pH 7.5 by using NaOH solution
➢ 50x TAE buffer	- 242 g Trizma - 57.1 mL Glacial acetic acid - 100 mL EDTA (0.5 M, pH 8) → fill up to 1000 ml with Ampuwa
➢ 1x TAE buffer	→ dilute 50x TAE buffer 1:50
➢ SYBR-Green stain solution	- 20 µl SYBR-Green stock solution - 35 ml 1x TAE buffer → storage under protection from light
➢ Electrophoresis solution	- 75 ml 2 M NaOH - 1 ml 0.5 M EDTA - 0.79 g Trizma → fill up to 500 ml with Ampuwa and adjust to pH 12.7 by using HCl

7.2 Supplementary results

7.2.1 Chromatograms of the intracellular arsenic speciation and quantification

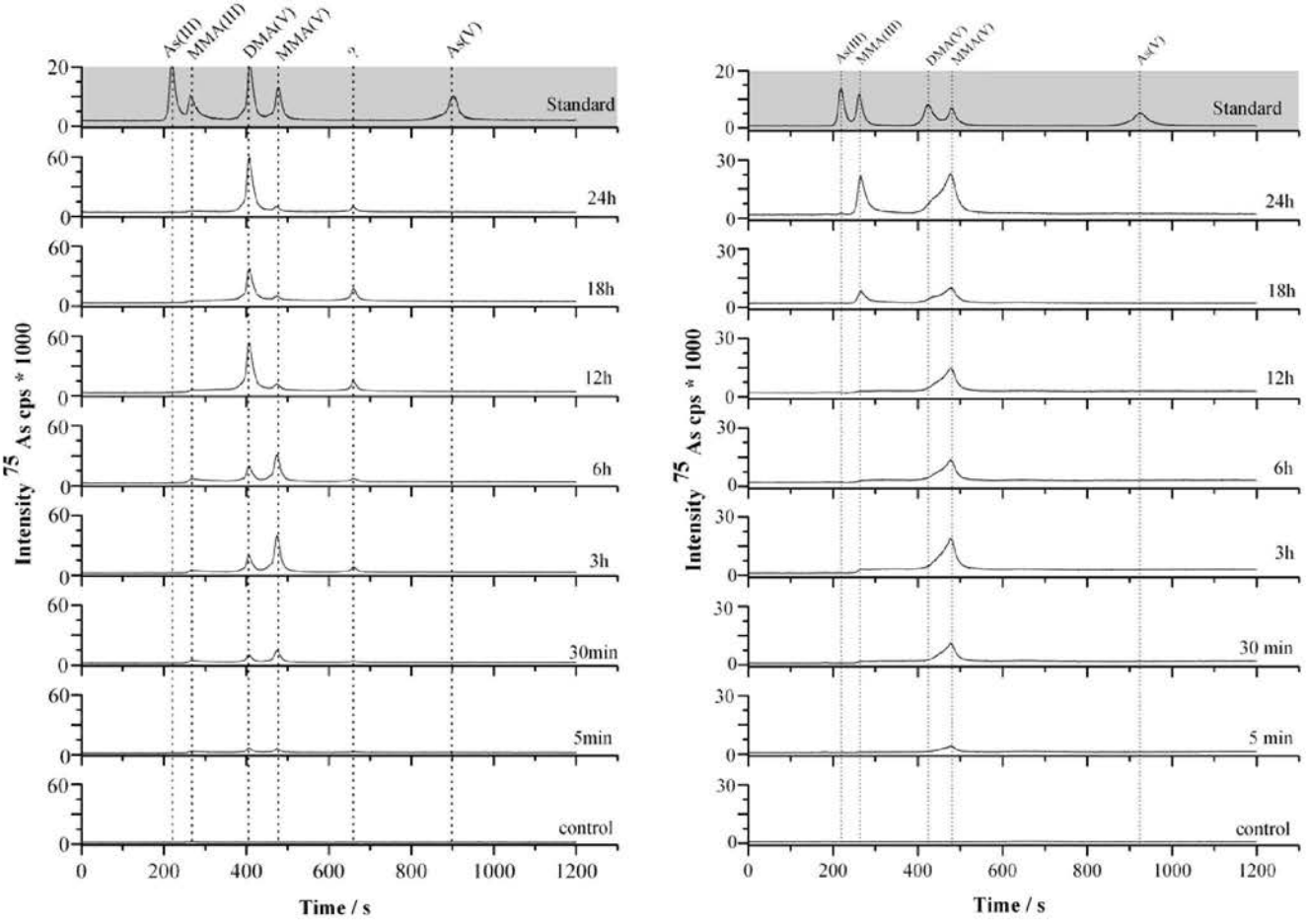

Fig. 56 Chromatograms of the intracellular arsenic speciation and quantification in the soluble fractions of (left) HepG2 cells and (right) of T24 cells

(Hippler et al., 2011)

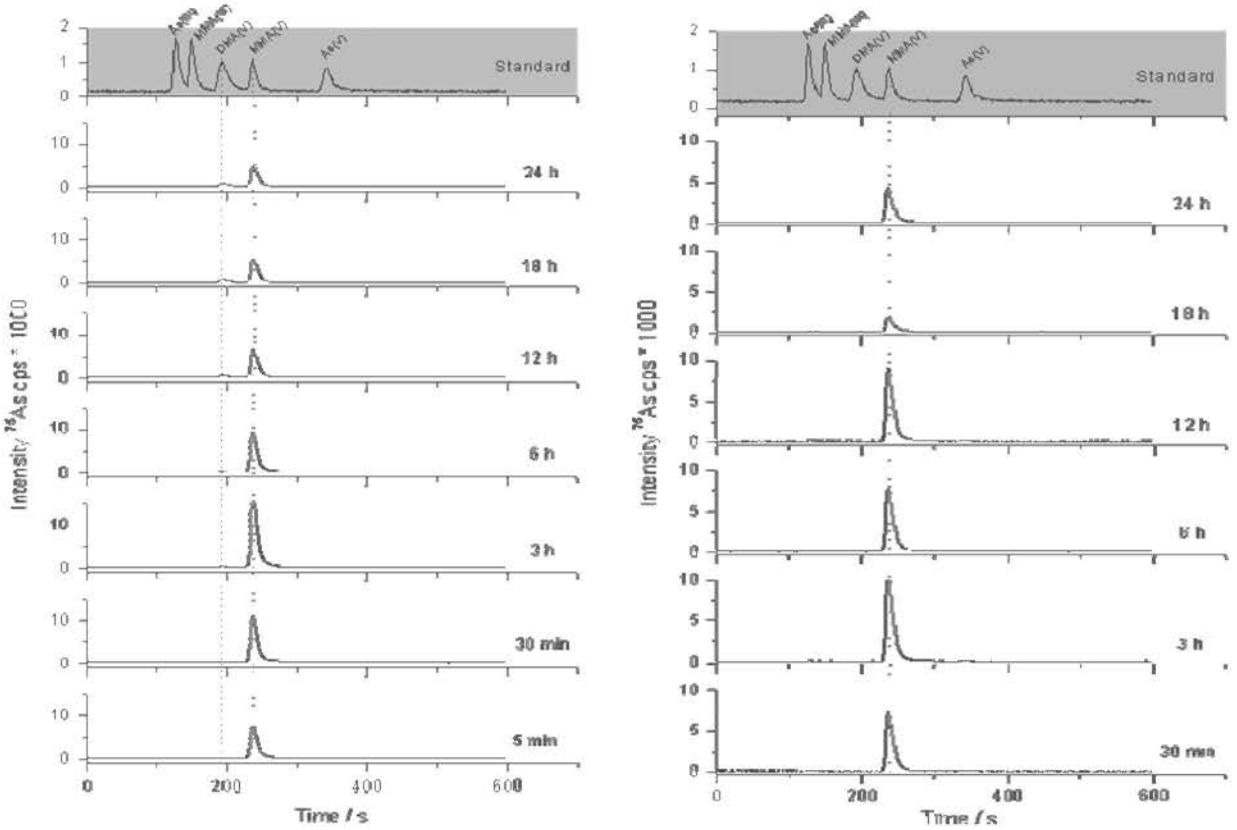

Fig. 57 Chromatograms of the intracellular arsenic speciation and quantification in the non-soluble fractions of (left) HepG2 cells and (right) T24 cells.

7.2.2 Data table of genotoxicity

Tab. 7 Results of the Alkaline Comet Assay with HUEPC cells (30 min of exposure)

DNA damage caused by MMA(III) was investigated by using the Alkaline Comet Assay after 30 min of exposure in HUEPC cells (mean ± standard error). Significance was tested by using the non-parametric Man Whitney Test with ns = no significant increase, * = p-value ≤ 0.05, ** = p-value ≤ 0.01,
***** = p-value ≤ 0.001, ct. = cytotoxic.**

	MMA(III)
negative control	0.748 ± 0.169
0.5 µM	0.896 ± 0.243 ns
5 µM	1.486 ± 0.365 *
50 µM	2.283 ± 1.406 ct.
ENU 0.1 mg / ml	1.996 ± 0.346 **

Tab. 8 Results of the Alkaline Comet Assay with HepG2 cells (30 min of exposure)

DNA damage caused by inorganic and methylated arsenic compounds was investigated by using the Alkaline Comet Assay after 30 min of exposure in HepG2 cells (mean ± standard error). Significance was tested by using the non-parametric Man Whitney Test with ns = no significant increase,
*** = p-value ≤ 0.05, ** = p-value ≤ 0.01, *** = p-value ≤ 0.001, ct. = cytotoxic.**

	As(III)	As(V)	MMA(III)	MMA(V)	DMA(V)	TMAO
negative control	0.321 ± 0.059	0.229 ± 0.035	0.322 ± 0.067	0.207 ± 0.036	0.159 ± 0.016	0.299 ± 0.037
0.1 µM	0.346 ± 0.045 ns	0.206 ± 0.029 ns	0.256 ± 0.034 ns	0.340 ± 0.050 **	0.166 ± 0.018 ns	0.230 ± 0.032 ns
0.5 µM	0.285 ± 0.045 ns	0.360 ± 0.062 ns	0.342 ± 0.067 ns	0.153 ± 0.015 ns	0.125 ± 0.015 ns	0.330 ± 0.060 *
1 µM	0.188 ± 0.030 ns	0.205 ± 0.024 ns	0.234 ± 0.028 ns	0.192 ± 0.020 ns	0.188 ± 0.020 ns	0.278 ± 0.043 ns
5 µM	0.304 ± 0.046 ns	0.175 ± 0.018 ns	0.330 ± 0.050 ***	0.142 ± 0.014 ns	0.122 ± 0.013 ns	0.346 ± 0.060 ns
10 µM	0.192 ± 0.017 ns	0.200 ± 0.022 ns	0.652 ± 0.066 ***	0.157 ± 0.014 ns	0.152 ± 0.017 ns	0.215 ± 0.028 ns
50 µM	0.495 ± 0.058 **	0.201 ± 0.023 ns	0.438 ± 0.037 ***	0.144 ± 0.014 ns	0.163 ± 0.020 ns	0.398 ± 0.074 ns
ENU 0.1 mg/ml	2.034 ± 0.201 ***	2.124 ± 0.197 ***	2.088 ± 0.199 ***	1.872 ± 0.208 ***	1.871 ± 0.209 ***	1.912 ± 0.207 ***

Tab. 9 Results of the Alkaline Comet Assay with T24 cells (30 min of exposure)

DNA damage caused by inorganic and methylated arsenic compounds was investigated by using the Alkaline Comet Assay after 30 min of exposure caused (mean ± standard error of mean). Significance was tested by using the non-parametric Man Whitney Test with ns = no significant increase, * = p-value ≤ 0.05,
**** = p-value ≤ 0.01, *** = p-value ≤ 0.001, ct. = cytotoxic, – = not tested.**

	As(III)	As(V)	MMA(III)	MMA(V)	DMA(III)	DMA(V)	TMAO
negative control	0.073 ± 0.009	0.093 ± 0.011	0.099 ± 0.010	0.080 ± 0.008	0.231 ± 0.023	0.085 ± 0.009	0.064 ± 0.008
0.001 µM	-	-	-	-	0.282 ± 0.025 ns	-	-
0.005 µM	-	-	-	-	0.339 ± 0.027 ***	-	-
0.01 µM	-	-	-	-	0.357 ± 0.027 ***	-	-
0.05 µM	-	-	-	-	0.395 ± 0.024 ***	-	-
0.1 µM	0.290 ± 0.032 ***	0.120 ± 0.016 ns	0.103 ± 0.013 ns	0.104 ± 0.011 ns	0.669 ± 0.034 ***	0.110 ± 0.011 ns	0.091 ± 0.009 ns
0.5 µM	0.272 ± 0.036 ***	0.129 ± 0.016 ns	0.154 ± 0.020 ns	0.098 ± 0.011 ns	ct.	0.069 ± 0.009 ns	0.094 ± 0.011 ns
1 µM	0.244 ± 0.033 ***	0.142 ± 0.019 ns	0.184 ± 0.025 ns	0.081 ± 0.010 ns	-	0.085 ± 0.010 ns	0.084 ± 0.010 ns
5 µM	0.256 ± 0.029 ***	0.138 ± 0.016 ns	0.129 ± 0.017 ns	0.084 ± 0.011 ns	-	0.064 ± 0.008 ns	0.084 ± 0.009 ns
10 µM	0.423 ± 0.044 ***	0.127 ± 0.014 ns	0.255 ± 0.025 ***	0.092 ± 0.010 ns	-	0.099 ± 0.012 ns	0.070 ± 0.009 ns
50 µM	0.282 ± 0.027 ***	0.344 ± 0.037 ***	0.550 ± 0.037 ***	0.103 ± 0.011 ns	-	0.090 ± 0.011 ns	0.107 ± 0.017 ns
ENU 0.1 mg/ml	0.949 ± 0.049 ***	0.984 ± 0.041 ***	1.116 ± 0.045 ***	0.932 ± 0.045 ***	ct.	1.044 ± 0.039 ***	1.267 ± 0.058 ***

Tab. 10 Results of the Alkaline Comet Assay with T24 cells (60 min of exposure)

DNA damage caused by inorganic and methylated arsenic compounds was investigated by using the Alkaline Comet Assay after 60 min of exposure in T24 cells (mean ± standard error). Significance was tested by using the non-parametric Man Whitney Test with ns = no significant increase,
*** = p-value ≤ 0.05, ** = p-value ≤ 0.01, *** = p-value ≤ 0.001, ct. = cytotoxic, – = not tested.**

	As(III)	As(V)	MMA(III)	MMA(V)	DMA(III)	DMA(V)	TMAO
negative control	0.105 ± 0.011	0.111 ± 0.012	0.145 ± 0.017	0.106 ± 0.012	0.158 ± 0.016	0.130 ± 0.014	0.122 ± 0.015
0.001 µM	-	-	-	-	0.210 ± 0.019 *	-	-
0.005 µM	-	-	-	-	0.167 ± 0.017 ns	-	-
0.01 µM	-	-	-	-	0.222 ± 0.018 **	-	-
0.05 µM	-	-	-	-	0.559 ± 0.031 ***	-	-
0.1 µM	0.150 ± 0.017 ns	0.169 ± 0.017 *	0.133 ± 0.012 ns	0.139 ± 0.014 ns	1.078 ± 0.189 ct.	0.151 ± 0.014 ns	0.119 ± 0.012 ns
0.5 µM	0.181 ± 0.019 *	0.158 ± 0.016 ns	0.121 ± 0.011 ns	0.185 ± 0.018 ***	ct.	0.165 ± 0.015 *	0.105 ± 0.011 ns
1 µM	0.187 ± 0.020 **	0.136 ± 0.018 ns	0.147 ± 0.017 ns	0.156 ± 0.014 *	-	0.153 ± 0.015 ns	0.120 ± 0.011 ns
5 µM	0.186 ± 0.017 **	0.184 ± 0.018 **	0.132 ± 0.013 ns	0.116 ± 0.011 ns	-	0.155 ± 0.014 ns	0.138 ± 0.015 ns
10 µM	0.165 ± 0.014 **	0.179 ± 0.016 ***	0.350 ± 0.038 ***	0.123 ± 0.015 ns	-	0.155 ± 0.015 ns	0.124 ± 0.012 ns
50 µM	0.182 ± 0.014 ***	0.168 ± 0.015 **	0.560 ± 0.034 ***	0.141 ± 0.014 ns	-	0.161 ± 0.014 ns	0.132 ± 0.014 ns
ENU 0.1 mg/ml	1.054 ± 0.047 ***	0.956 ± 0.041 ***	1.236 ± 0.075 ***	1.199 ± 0.060 ***	ct.	1.311 ± 0.077 ***	1.536 ± 0.066 ***

7.3 Supplementary material

7.3.1 Curriculum Vitae

The CV is not included in the online version for reasons of data protection.

The CV is not included in the online version for reasons of data protection.

The CV is not included in the online version for reasons of data protection.

- Annex -

The CV is not included in the online version for reasons of data protection.

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7.3.2 Declarations

Versicherung an Eides Statt

Ich, Ricarda Zdrenka, Zum Ravenhorst 298, 46147 Oberhausen, Matr.-No. 1529137,

versichere an Eides Statt durch meine Unterschrift, dass ich die vorstehende Arbeit selbständig und ohne fremde Hilfe angefertigt und alle Stellen, die ich wörtlich oder annähernd wörtlich aus Veröffentlichungen entnommen habe, als solche kenntlich gemacht habe. Dies betrifft auch Passagen, die aus meiner eigenen Publikation enstammen (Zdrenka et al., 2012[4]) und in der vorliegenden Arbeit größtenteils wörtlich übernommen wurden. Ich habe mich keiner anderen als der angegebenen Literatur oder sonstiger Hilfsmittel bedient.

Ich versichere an Eides Statt, dass ich die vorgenannten Angaben nach bestem Wissen und Gewissen gemacht habe und dass die Angaben der Wahrheit entsprechen und ich nichts verschwiegen habe.

Die Strafbarkeit einer falschen eidesstattlichen Versicherung ist mir bekannt, namentlich die Strafandrohung gemäß § 156 StGB bis zu drei Jahren Freiheitsstrafe oder Geldstrafe bei vorsätzlicher Begehung der Tat bzw. gemäß § 161 Abs.1 StGB bis zu einem Jahr Freiheitsstrafe oder Geldstrafe bei fahrlässiger Begehung.

Oberhausen, 6.8.2013 — Ricarda Zdrenka

Ort, Datum — Unterschrift

[4] Zdrenka R, Hippler J, Johnen G, Hirner AV & Dopp E (2012) Intracellular Arsenic Speciation and Quantification in Human Urothelial and Hepatic Cells. In: Bladder Cancer - From Basic Science to Robotic Surgery, Canda AE (Ed.), ISBN: 978-953-307-839-7, InTech

Erklärung

der
(1) Dipl.-Chem. cand. Dr. rer. nat. Joerg Hippler, Institute of Environmental Analytical Chemistry, University of Duisburg-Essen, Essen, Germany
(2) Dr. Georg Johnen, Institute for Prevention and Occupational Medicine of the German Social Accident Insurance, Institute of the Ruhr University Bochum (IPA), Bochum, Germany
(3) Prof. Dr. Alfred V. Hirner, Institute of Environmental Analytical Chemistry, University of Duisburg-Essen, Essen, Germany
(4) Prof. Dr. Elke Dopp, Zentrum für Wasser- und Umweltforschung, University of Duisburg-Essen, Essen, Germany

zur Dissertation der Frau Dipl.-Chem. cand. Dr. rer. nat. Ricarda Zdrenka:

"EARLY MOLECULAR CHANGES IN ARSENIC EXPOSED HUMAN UROTHELIAL CELLS DEPENDING ON CELLULAR UPTAKE AND BIOTRANSFORMATION"

vorgelegt an der Universität Duisburg-Essen, Fachbereich Chemie

1. Die v.g. Unterzeichner zu (1) bis (4) sind Co-Autoren der von Frau Dipl.-Chem. cand. Dr. rer. nat. Ricarda Zdrenka u.a. im Buch: "Bladder Cancer - From Basic Science to Robotic Surgery" edited by Abdullah Erdem Canda im Januar 2012 zu Part 20, Seite 405-428 verfassten und veröffentlichten Abhandlung: "Intracellular Arsenic Speciation and Quantification in Human Urothelial and Hepatic Cells".

2. Die Unterzeichner haben z.T. folgende eigene Forschungsergebnisse, wie Ergebnisse zur intrazellulären Arsen-Speziierung und –Quantifizierung sowie zur miRNA-Analyse und DNA-Methylierung erarbeitet und diese u.a. der vorgenannten Abhandlung beigesteuert.
   Sie sind erlaubterweise auch in der Dissertation an den entsprechenden Stellen der Kapiteln 3 „Material und Methods“ und 4 „Results“ von Frau Dipl.-Chem. cand. Dr. rer. nat. Ricarda Zdrenka verarbeitet und kenntlich gemacht worden.

3. Die Unterzeichner haben ihre Forschungsergebnisse insoweit untereinander und zusammen mit Frau Dipl.-Chem. cand. Dr. rer. nat. Ricarda Zdrenka diskutiert und deren eigenständiger wissenschaftlicher Ergebnisfindung unterstellt.

4. Frau Dipl.-Chem. cand. Dr. rer. nat. Ricarda Zdrenka hat daher unsere Forschungsergebnisse und Diskussionsbeiträge ihrer eigenen wissenschaftlichen Würdigung unterzogen und daher das Ergebnis ihrer

eigenen Forschungsarbeit in der vorgenannten Abhandlung wie auch Dissertation selbstständig niedergelegt und verarbeitet.

Essen, 15.05.13	
(Ort, Datum)	(Name)
Essen, 17.5.13	
(Ort, Datum)	(Name)
Essen, den 15.5.13	
(Ort, Datum)	(Name)
Bochum, 29.5.13	
(Ort, Datum)	(Name)

7.3.3 Acknowledgement

Help and Caring
Thanks for doing what you did;
You are kind beyond belief;
Your help and caring calmed me down,
And gave me soothing relief.

By Karl and Joanna Fuchs

First of all I owe my deepest gratitude to my supervisor Prof. ElkeDopp and the Institute of Hygiene and Occupational Medicine. Without your excellent academic guidance, intellectual inspirations, and continued encouragement, I would have hardly been where I am today. Thank you for everything!

I am deeply grateful to Prof. Alfred V. Hirner at the Institute of Environmental Analytical Chemistry and Dr. Georg Johnen at the Institute for Prevention and Occupational Medicine of the German Social Accident Insurance for their cooperation and for giving me the opportunity to work and learn in their institutes. Without your and your colleagues' continuous support, advice, and valuable discussions throughout the course of this investigation, this study would hardly have been completed. Thus, a special thank goes to Jörg Hippler at the Institute of Environmental Analytical Chemistry, Dr. Daniel G. Weber, Dr. Oleksandr Bryk, Peter Rozynek, Yvonne von der Gathen and Irina Raiko at the Institute for Prevention and Occupational Medicine of the German Social Accident Insurance, and Dr. Christian Johannes at the Department of Genetic for their invaluable support and their continued encouragement. Thank you all so much!

I am indebted to all of my colleagues at the Institute of Hygiene of Occupational Medicine. I owe my deepest thanks especially to Ms. G. Zimmer, and Mrs. U. Zimmermann for providing me with proper guidance during my experiments. Very special thanks go to my colleagues and friends Melanie Gerhards, Jessica Richard, Nisha Verma and Mario Pink for all the help and the work we have done together, the thought-provoking and fruitful discussions, and the fun we had together. Thank you all for this wonderful and unforgettable time!

I would like to show my gratitude to the German Research Foundation (DFG), the Centre of Water- and Environmental Research (ZWU) and the Faculty of Chemistry of the University of Duisburg-Essen, and the German Chemical Society (GDCh) for their generous financial support, which allowed me to elaborate this project and to present my results to professional audience on international scientific conferences.

I would also like to thank my dearest friend Maia Becker, as well as my colleagues at Dr. Knoell Consult GmbH, especially Dr. Jasmina Oulmi-Kagermann , Dr. Gisela Werle-Schneider, and Dr. Monica Sica, for reviewing the manuscript of this thesis and for their continuous support and encouragement to finalise this work. Thank you so much!

It is an honour for me to thank my family and friends, who always believed in me and encouraged me. My dearest thanks go to my parents, who gave all their support, confidence and love. Without you, I would not be where I am today. This thesis is dedicated to you. Thank you for everything!