Toxins in Renal Disease and Dialysis Therapy: Genotoxic Potential and Mechanisms

Dissertation zur Erlangung des
naturwissenschaftlichen Doktorgrades der
Bayerischen Julius-Maximilians-Universität Würzburg

vorgelegt von
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Würzburg, 2008

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Tag des Promotionskolloquiums: …………………………………

Doktorurkunde ausgehändigt am: …………………………………

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A Introduction

1 Kidney and Kidney Disease

1.1 Kidney Anatomy and Function of Kidneys

1.1.1 Macroscopic Organisation

The kidneys are two bean-shaped organs, which are part of the urinary system (Fig. A-1). The concave side of the kidney contains an opening – the hilum - which admits the renal vein, artery, nerves and the ureter. In humans the kidneys are located on both sides of the spine just below the diaphragm. They are enclosed in a fibrous renal capsule and embedded in adipose tissue, which absorbs shocks. The structure of the kidney consists of two parts: (1.) the outer part – the renal cortex – and (2.) the inner part – the renal medulla. The renal medulla is composed of 10 - 20 renal pyramids. Each pyramid conjoined with the cortex forms a renal lobe. The tip of each pyramid - the renal papilla – empties into a calyx, which forms the beginning of the urinary tract.

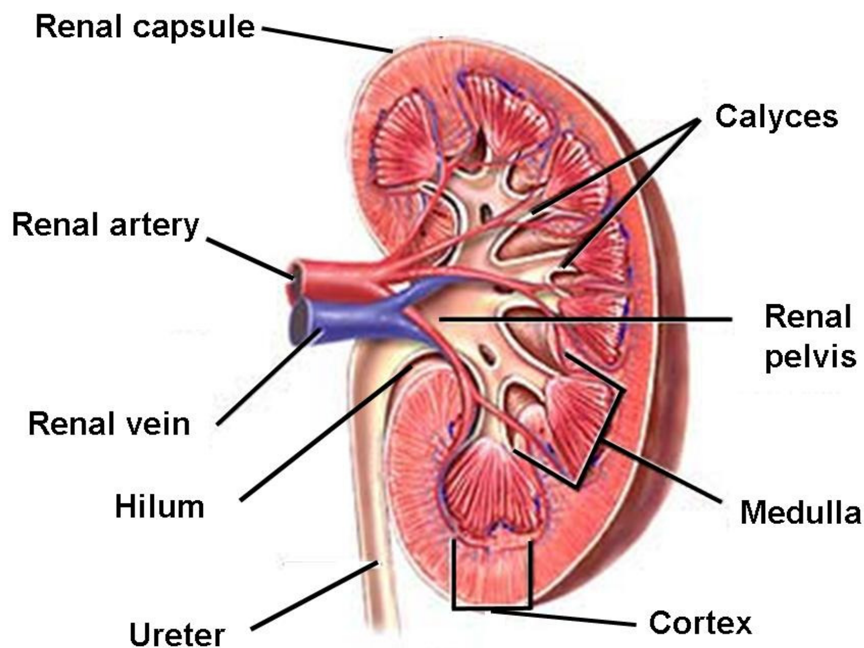


Fig. A-1 Kidney anatomy (adapted from MedLinePlus, 2007)

1.1.2 Microscopic Organisation and Function

The basic functional unit of the kidney is the nephron. More than one million nephrons per kidney are located within cortex and medulla. They consist of a filtering component - the renal corpuscle – and a part specialized in reabsorption and secretion – the renal tubule. The renal corpuscle is composed of the glomerulus and the Bowman's capsule. The glomerulus is a capillary tuft through which blood flows under pressure. The pressure forces water and small solutes of the blood to be filtered through the capillary walls into the Bowman's capsule, thereby forming the nephric filtrate. The nephric filtrate flows into the renal tubule, which consists of several sections: the proximal tubule, the loop of Henle and the distal convoluted tubule. In these sections organic solutes like glucose and amino acids, most of the water and salts are reabsorbed, while other substances like hydrogen or ammonium are excreted actively. Finally, urine flows through the collecting duct system, is drained into the bladder via the ureter and finally excreted.

By producing urine the kidneys fulfil their main functions: excreting metabolic waste products and maintaining the homeostasis of the organism. While keeping the homeostasis, the kidneys also regulate the acid-base balance, the blood pressure and the plasma volume.

1.2 Kidney Failure

Generally, humans can live with reduced kidney function or even with a single kidney. However, several diseases can threaten the health of a person because they result in a dramatically diminished kidney function. On one hand there is acute renal failure, which develops within hours or days and is generally reversible. Reasons for acute renal failure are e.g. infections, hypotension, medication or kidney stones. On the other hand there is the slowly progressing disease of chronic renal failure (CRF). The leading cause for CRF in the western world is diabetes mellitus (US Renal Data System 2004), followed by high blood pressure and glomerolunephritis, while in third world countries HIV infection also plays a important role (Lu and Ross 2005).

After years of suffering from CRF, the glomerula filtration rate of the kidney finally drops below 15%, leading to end-stage renal disease (ESRD). When ESRD is reached, renal replacement therapy, like renal transplantation or hemodialysis is necessary. In the beginning of 2006 more than 64,000 patients in Germany depended on hemodialysis. Due to demographic changes, increased prevalence of

diabetes and higher life expectancy of ESRD patients, the number of dialysis patients increases about 4.8% per year and will reach 100,000 within the next few years (Frei and Schober-Halstenberg 2006).

Of course, this problem is not limited to Germany. For the USA a 32% increase of dialysis patients is expected between 2000 and 2015 (Gilbertson, Liu et al. 2005), and 2 million ESRD patients world-wide are expected by 2010 (Lynsaght 2002). Due to the lack of donor kidneys most patients will have to be treated by hemodialysis.

1.3 Renal Replacement Therapies

The method of artificial kidney replacement is called dialysis: This method does not heal the underling kidney disease, but it allows removal of waste products – the so called uremic toxins (see page 19) - and excess fluid from the blood of the patient. Two dialysis methods are currently available: peritoneal dialysis and hemodialysis (HD). HD is the most frequent renal replacement therapy with about 180 million applications worldwide per year.

1.3.1 Hemodialysis

In HD, the arterial blood is pumped from the fore-arm vein of a patient through tubes into the blood compartment of a dialyser, where it flows through ca. 10,000 hollow fibers with walls of semipermeable membranes. On the other side of the membranes, a dialysis solution is pumped in counter current flow through the dialysate compartment of the dialyser (Fig. A-2), allowing the diffusion of waste products from the blood-compartment into the dialysis fluid compartment. In order to enhance the natural diffusion alongside the concentration gradient, blood is pumped at 250 - 300 ml/min while the dialysis fluid flows at 500 ml/min. The semipermeable membrane contains pores large enough to allow water and uremic toxins to pass across. After flowing through the dialyser the dialysis fluid is discharged while the cleansed blood is pumped back into the body. In general HD is performed three times a week for 4 - 5 h.

More efficient techniques for HD are hemodiafiltration and hemofiltration where a convective flow in the sense of solvent drag is applied. Hemodiafiltration and hemofiltration are applied preferentially when larger toxin molecules are removed, i.e. peptides or small proteins with MW of > 10,000 Da.

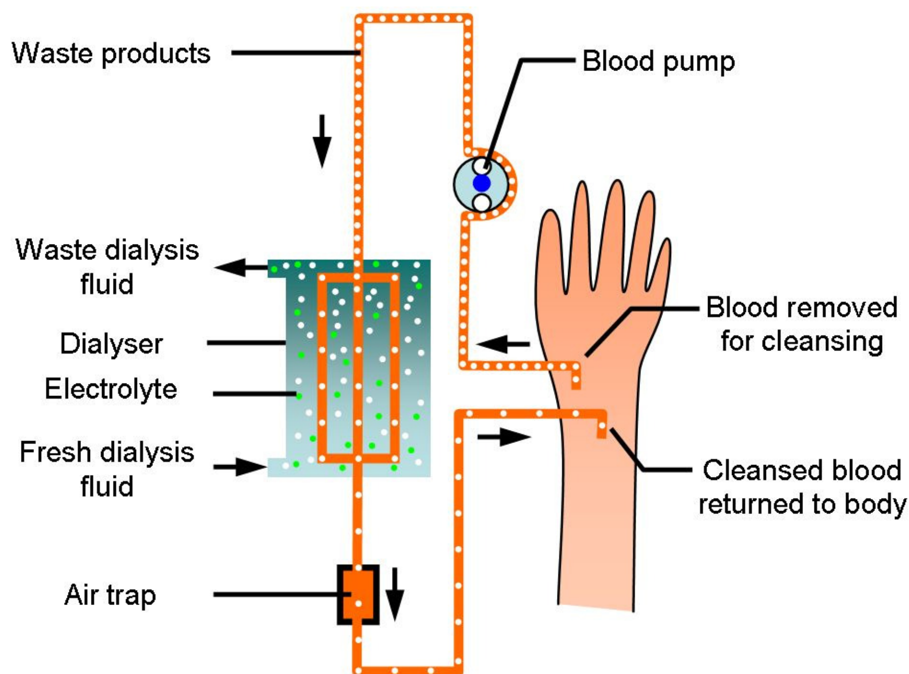


Fig. A-2 Schematic picture of a hemodialysis circuit

1.4 Hemodialysers

Currently a wide spectrum of hemodialysers combined with different membranes is available (Tab. A-1). Generally, membranes are produced by two families of polymers: synthetic and cellulosic. These classes of membranes can be subdivided into high-flux membranes (large pores) or low-flux membranes (small pores). High flux membranes allow higher water flux and better removal of high molecular weight uremic solutes than low flux membranes (Boure and Vanholder 2004).

The polymer of the membrane determines the physical, chemical and biological properties of a dialysis membrane. Ideally a membrane is highly biocompatible, adsorbs dialysate impurities from the dialysis fluid, removes middle molecules and is resistant to all chemical and sterilizing agents used in HD procedures (Boure and Vanholder 2004; Uhlenbusch-Körwer, Bonnie-Schorn et al. 2004). Given that synthetic membranes are superior to cellulosic membranes in most of these properties - especially in biocompatibility – there is a trend towards synthetic polymer material (Vienken and Bowry 2002).

Celluliosic		**Synthetic**	
Unmodified (low-flux)	Modified/ regenerated (high-flux):	Low-flux	High-flux
• Cuprammonium rayon • Cellulose diacetate	• Cellulose triacetate	• Polysulfone • Polycarbonate	• Polysulfone • Polyamide • Polyamide and Polysulfone blends • Polyethersulfone • Polyacrylonitrile • Polymethyl-methacrylate

Tab. A-1 Types of membranes with examples (not exhaustive); (Boure and Vanholder 2004):

1.5 Problems caused by Dialysis

Unfortunately, HD treatment can also produce side-effects. Common problems are: (1.) the “first-use syndrome”- an allergic reaction towards materials of medical devices or residues of sterilisation (Charoenpanich, Pollak et al. 1987), (2.) bacterial or endotoxin contamination by improper treatment of water, dialysate, dialysis machines and dialysers (Nicholls and Platts 1985; Gordon, Drachman et al. 1990; Pegues, Beck-Sague et al. 1992; Burwen, Olsen et al. 1995) and (3) contamination by leachable degradation products of dialyser membranes (Lucas, Kalson et al. 2000).

1.6 Dialysis Patients and Cancer

On top of these problems dialysis patients are at increased risk of cancer, especially cancer of the urinary tract (Maisonneuve, Agodoa et al. 1999; Teschner, Garte et al. 2002; Stewart, Buccianti et al. 2003; Vajdic, McDonald et al. 2006) (Tab. A-2). The risk of kidney cancer rises significantly with time on dialysis (Stewart, Buccianti et al. 2003). The risk is also higher in young than in old patients and higher in females compared to males (Stewart, Buccianti et al. 2003).

Site	SIR (95% confidence interval)		
	Australia and New Zealand	Europe	USA
All but skin	1.8 (1.7 - 2.0)	1.1 (1.0 - 1.1)	1.2 (1.2 - 1.2)
Oral cavity	1.4 (0.9 - 2.4)	0.6 (0.5 - 0.7)	1.3 (1.2 - 1.4)
Respiratory	1.5 (1.1 - 1.9)	0.9 (0.9 - 1.0)	1.1 (1.1 - 1.2)
Bone, skin, breast	1.4 (1.1 - 1.8)	1.0 (0.9 - 1.1)	0.8 (0.8 - 0.9)
Hemopoietic	1.6 (1.1 - 2.3)	1.3 (1.2 - 1.4)	2.5 (2.4 - 2.6)
Digestive	1.2 (1.0 - 1.5)	0.9 (0.9 - 1.0)	1.2 (1.2 - 1.3)
Genitourinary			
All	3.0 (2.6 - 3.5)	1.4 (1.4 - 1.4)	1.1 (1.1 - 1.1)
Bladder	4.8 (3.6 - 6.2)	1.5 (1.4 - 1.7)	1.4 (1.3 - 1.5)
Kidney	9.9 (7.7 - 12.3)	3.3 (3.1 - 3.6)	3.7 (3.5 - 3.9)
Other and unspecific			
All	2.3 (1.7 - 3.1)	1.1 (1.0 - 1.2)	2.2 (2.0 - 2.4)
Thyroid	5.9 (3.3 - 10.7)	1.9 (1.5 - 2.3)	2.4 (2.1 - 2.8)

Tab. A-2 Site-specific cancer risk in ESRD patients (Maisonneuve, Agodoa et al. 1999)

Several factors may contribute to the increased cancer incidence: chronic infections, a weakened immune system, pre-treatment with immunosuppressive drugs, nutritional deficiencies or the depressed DNA repair in CRF patients (Malachi, Zevin et al. 1993; Maisonneuve, Agodoa et al. 1999).

Other possible factors which could contribute are: (1) the accumulation of genotoxic uremic toxins in the blood of the patients or (2) substances leaching from extracorporal blood circuit into the blood of HD patients (e.g. bisphenol A or di(2-ethylhexyl) phthalate).

2 Substances Leaching from Extracorporeal Blood Circuit

During HD blood can be exposed to a variety of compounds derived from tubing, membranes, dialysis fluid and housing. Especially disconcerting are substances with known toxic properties or endocrine disrupting chemicals. The term endocrine disruptor is commonly used to describe environmental agents which alter the endocrine system, by interacting with hormone receptors (Reviewed by (McLachlan 2001)). Thereby, they can cause alterations in the hormone level leading to infertility, feminisation of males, reproductive tract malformation, endometriosis and tumours in estrogen-responsive tissues (McLachlan 2001; Wozniak, Bulayeva et al. 2005).

Those effects have preferentially been reported in animal models, e.g. the mouse model.

Two substances possessing those properties and known to leach from extracorporal blood circuits have raised special concern: **di(2-ethylhexyl)phthalate (DEHP) and bisphenol A (BPA)**.

2.1 Bisphenol A

2.1.1 Structure and Use

BPA is the common name for 2,2-(4,4-dihydroxy-diphenyl)propane (Fig. A-3). It is synthesized by condensation of two equivalents phenol with one equivalent acetone at low pH and high temperatures.

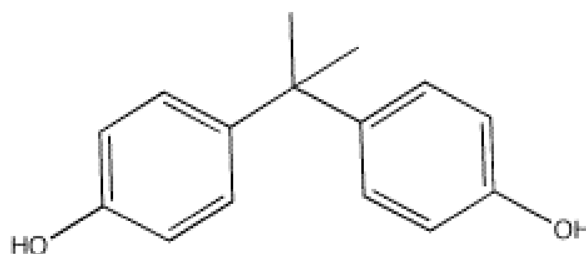


Fig. A-3 Molecular structure of bisphenol A

The main application of BPA is as monomer component for polycarbonate plastic and epoxy resins (Staples, Dorn et al. 1998). Consequently, it is used in numerous consumer products (e.g. food packaging) and dentistry (e.g. brackets, dental fillings). Unreacted BPA residues, or BPA resulting from hydrolysis of the ester bonds, can leach from plastics into food, water (Imai and Komabayashi 2000; Bae, Jeong et al. 2002; Lopez-Cervantes and Paseiro-Losada 2003; Sajiki and Yonekubo 2003; Sajiki and Yonekubo 2004), or saliva and is ingested (Suzuki, Ishikawa et al. 2000; Watanabe, Hase et al. 2001; Atkinson, Diamond et al. 2002; Watanabe 2004).

2.1.2 Exposure and Metabolism

While the major route of exposure is by food (Scientific committee on toxicology 2002), HD patients obtain an additional burden of BPA which leaches off the dialyser directly into the blood (Haishima, Hayashi et al. 2001; Yamasaki, Nagake et al. 2001; Murakami, Ohashi et al. 2007). This is important to notice because orally absorbed

BPA undergoes an extensive first-pass effect in the liver, resulting in the detoxified form of BPA: BPA-glucuronide. When BPA leaches directly into the blood (or is applied *i.p.* in test animals) more of the highly bioavailable, free BPA circulates and stronger effects can be the result (Scientific committee on toxicology 2002). Fortunately, BPA has no tendency to accumulate and a half-life of less than one day was described (Pottenger, Domoradzki et al. 2000; Takahashi and Oishi 2000).

Following oral exposure BPA is rapidly absorbed from the gastrointestinal tract in rodents. However, it is not possible to quantify the actual extent of absorption because the major route of excretion is *via* the faeces as parent BPA (50 - 80% depending on species, strain and gender). The parent BPA can result from two different sources: It is either 1.) BPA which passes the intestinal tract unchanged and is not absorbed or 2.) its glucuronide form which is transported into the intestine *via* bile and is hydrolysed later on. The excretion route of secondary importance is *via* the urine in the form of BPA-glucuronide. Ten additional metabolites could be detected in urine of mice but nearly no unmodified BPA (Pottenger, Domoradzki et al. 2000; Snyder, Maness et al. 2000; Elsby, Maggs et al. 2001; EU-Report 2003; Zalko, Soto et al. 2003).

Nevertheless, parent BPA could be detected in the serum of pregnant females by derivatisation - GC/MS ranging from 0.3 to 18.9 ng/ml (Schönfelder, Wittloht et al. 2002). BPA could even be detected in foetal plasma (0.2 - 9.2 ng/ml). Several other studies detected between 0.32 and 2.59 ± 5.23 ng/ml BPA in plasma, though mostly by less sensitive detection methods (for review see (Welshons, Nagel et al. 2006)). These values are in line with BPA levels in urine: 0.04 µg/l – 8 µg/l (Calafat, Kuklenyik et al. 2005).

2.1.3 *In vitro* and *In vivo* Effects of BPA

2.1.3.1 Acute Toxicity

The acute toxicity test for BPA determined oral LD_{50} values above 2,000 mg/kg bw for laboratory animals (NTP 1982). Since the BPA levels in nature and humans are far lower, it can be concluded that the acute toxicity is not of concern for humans. Therefore research focus has focussed on possible carcinogenic/mutagenic and estrogenic effects.

2.1.3.2 Reproductive and Developmental Toxicity

Until recently BPA has generally been considered a relatively weak estrogen. Depending on the test system, the binding affinity of BPA to estrogen receptor α (ER-α) or β is 2 - 4 orders of magnitude lower than that of 17β estradiol (Feldman and Krishnan 1995; Dodge, Glasebrook et al. 1996; Kuiper, Lemmen et al. 1998; Maruyama, Fujimoto et al. 1999). The glucuronide shows even less estrogenic activity (Snyder, Maness et al. 2000; Matthews, Twomey et al. 2001). However, a recent study showed that BPA not only acts *via* genomic responses of the ER, but also through non-genomic membrane-initiated pathways (Wozniak, Bulayeva et al. 2005). Nanomolar concentrations of BPA resulted in an increased Ca^{2+} influx *in vitro*. This may lead to changes in the signalling process or hormone secretion.

In vivo BPA is 10,000 fold less potent in inducing uterotrophic effects in ovariectomized rats than 17β-estradiol (Milligan, Balasubramanian et al. 1998). An uterotropic effect in rats was confirmed after high oral or subcutaneous dosing by several other groups (Ashby and Tinwell 1998; Laws, Carey et al. 2000; Matthews, Twomey et al. 2001; Ashby and Odum 2004).

To elucidate the relevance of these observations regarding reproductive toxicity, an elaborate three generation study on CD Sprague-Dawley rats was conducted (Tyl, Myers et al. 2002). BPA dosage of up to 5 mg/kg bw per day did not cause any effects. Reproductive toxicity could only be observed at concentrations which also produced systemic toxicity (> 50 mg/kg bw per day). This was in line with an earlier study, which also concluded that BPA is no selective reproductive toxicant (no effect up to 640 mg/kg bw per day) (Morrissey, George et al. 1987). Tinwell *et al* also detected reduced sperm count and a delay in the day of vaginal opening in Alderlay Park rats only at high doses (50 mg/kg bw per day) (Tinwell, Haseman et al. 2002).

In contrast to those high-dose studies, several groups report effects already at a low-dose level in mice. While there is no dispute on the high-dose effects, low dose effects are less clear, especially because many of the reported effects could not be reproduced in large animal trials.

Low oral doses of BPA (2 - 20 µg/kg bw per day) have been reported to affect male reproductive organs such as preputial glands and epididymides and to reduce sperm production (Nagel, vom Saal et al. 1997; vom Saal, Timms et al. 1997; vom Saal, Cooke et al. 1998). However, other groups could not confirm these observations; even though the same animal strains were used (Ashby, Tinwell et al.

1999) and additional doses were tested (Cagen, Waechter et al. 1999; Cagen, Waechter et al. 1999). Low-dose oral BPA administration during gestation had no adverse effect on female offspring in regard to rat puberty development and reproductive functions in CF1 mice (20 µg/kg/day) (Ashby and Tinwell 1998), or female and male SD rats (3 mg/kg bw per day) (Nagao, Saito et al. 1999). *In utero* exposure of SD rats and Alderlay Park rats (20 µg/kg bw – 100 µg/kg bw) did not influence litter size, weight, anogenital distance at birth, first estrus, days of vaginal opening or weight of reproductive organs.

Even though the discussion about the low-dose effect is still ongoing it might be of relevance for humans: a small preliminary study on Japanese women (n = 77) found a correlation between plasma BPA level and subsequent miscarriages (Sugiura-Ogasawara, Ozaki et al. 2005).

2.1.3.3 Mutagenicity

Another concern about constant low-dose BPA exposure is the possible carcinogenic mutagenic capacity of BPA. Two studies detected DNA adduct formation following BPA incubation with purified DNA (Atkinson and Roy 1995; Atkinson and Roy 1995). However, the adduct formation was distinctly decreased in the presence of inhibitors of cytochrom P450. Therefore the relevance for the *in vivo* situation is uncertain. Additionally BPA inhibited microtubule polymerisation in cell free systems (Metzler and Pfeiffer 1995; Pfeiffer, Rosenberg et al. 1997)

In contrast to the results of cell-free systems, BPA was not mutagenic in the Ames test of variety of *Salmonella typhimurium* strains, with and without metabolic activation (Andersen, Kiel et al. 1978; Haworth, Lawlor et al. 1983; Tennant, Stasiewicz et al. 1986; Schweikl, Schmalz et al. 1998)

Studies in mammalian cells yielded mixed results. BPA was not mutagenic in mutation tests with mouse lymphoma L5178Y cells (Myhr and Caspary 1991), Chinese hamster V79 cells (Schweikl, Schmalz et al. 1998) and Syrian hamster embryo (SHE) cells (Tsutsui, Tamura et al. 1998).

However, it produced positive results in transformation assays in the same study (Tsutsui, Tamura et al. 1998). BPA (µM) caused aneuploidy in somatic cells (Tsutsui, Tamura et al. 2000) and induced micronuclei (MN) in V79 cells (Pfeiffer, Rosenberg et al. 1997). Furthermore, 100 - 200 µM BPA caused aberrant spindle formation in V79 cells (Ochi 1999) and *in vivo* in mice oocytes at environmentally relevant doses

(Hunt, Koehler et al. 2003; Susiarjo, Hassold et al. 2007). Even nanomolar concentrations had the effect of inducing proliferation of the human prostate cancer cell line LNCaP (Wetherill, Petre et al. 2002).

2.1.3.4 Carcinogenicity

A well conducted two-year carcinogenicity study of the US National Toxicology Program concluded that there is no convincing evidence for carcinogenic potential of BPA in B6C3F_1 mice (up to 5,000 ppm BPA for male mice approximately 833 mg/kg bw per day; up to 10,000 ppm BPA for female mice approximately 1666 mg/kg bw per day.) and F344 rats (up to 2,000 ppm BPA; approx. 100 mg/kg bw per day) (NTP 1982). However, there was a marginal but statistically significant increase in leukaemia in male rats, along with a not statistically significant increase in leukaemia in female rats. The male mice showed a marginal significant increase of lymphomas and leukemias.

Another study also found a marginal increase of leukaemia in F344 rats (2,000 ppm BPA approximately 100 mg/kg bw) and lymphoma, as well as leukaemia in low-dose (5,000 ppm BPA approximately 833 mg/kg bw) male B6C3F1 mice (Huff 2001). Therefore the authors concluded that an association of BPA exposure with increased cancers of the hematopoietic system cannot be ruled out.

Carcinogenesis studies on humans cannot be performed, but epidemiological studies on humans found no correlation between breast cancer incidence and BPA exposure in American women (Aschengrau, Coogan et al. 1998).

2.1.4 Concerns

With regard to these results the European Chemicals Bureau concluded that, although there is need for additional studies, BPA poses no risk for consumers (EU-Report 2003). The tolerable daily intake (TDI) was even raised from 0.01 mg/kg bw per day to 0.05 mg/kg bw per day by the EFSA panel (European Food Safety Authority) (EFSA 2006). BPA levels which normally absorbed are in the low µg/kg bw range. Therefore they are below the TDI range and the potential hazard for humans was regarded as minimal (EU-Report 2003). Solely workers handling BPA may exceed the TDI and may therefore be at risk. Another evaluation by Haighton et al. following the weight-of-evidence approach as advised by the Internation Agency for Research on Cancer (IARC) and US Environmental Protection Agency (US EPA) concluded that BPA is not likely to be carcinogenic to humans (Haighton, Hlywka et

al. 2002). Additional evaluation by the Scientific Committee on Toxicology, Ecotoxicity and the Environment (CSTEE) followed this argumentation (Scientific committee on toxicology 2002). However, this conclusion is challenged by other groups (Huff 2001; vom Saal and Hughes 2005).

As the debate is ongoing at the moment, the importance of additional information, especially regarding special risk populations like HD patients is obvious.

2.2 Phthalates

Phthalates or phthalate esters are a group of chemicals which share a common chemical structure: they are dialkyl or alkyl/alcaryl esters of 1, 2-benzenedicaroxylic acid. In Western Europe about one million tonnes of phthalates are produced annually. Most of it is used as plasticizers to impart flexibility to plastics (Intermediates 2007). One of the most widely used phthalates is di(2-ethylhexyl) phthalate (DEHP).

2.2.1 Structure and Use of Di(2-Ethylhexyl)phthalate

DEHP is also known as bis(2-ethylhexyl) phthalate (BEHP) or dioctyl phthalate (DOP) (Fig. A-4).

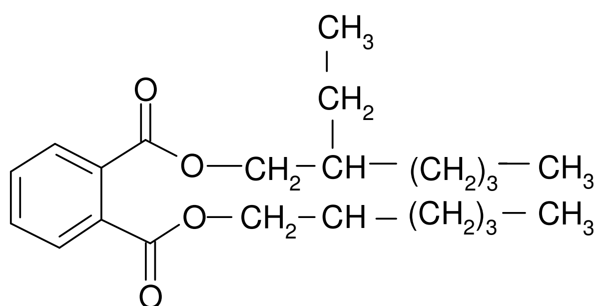


Fig. A-4 Molecular structure of di-(2-ethylhexyl)phthalate

DEHP is synthesized by esterification of phthalic acid anhydride with 2-ethylhexanol.

Its primary use is as plasticizer in PVC, e.g. in flooring and food storage containers, but it is also used in paints, lubricants and clothing. Another important application is in medical care products like blood bags, transfusion bags and tubings, catheters or air tubes (Calafat and McKee 2006; Umweltbundesamt 2006).

PVC can contain up to 40% DEHP. DEHP is not covalently bound to the plastic and can therefore leach or migrate from it. It is easily absorbed by aliphatic substances.

2.2.2 Exposure to and Metabolism of DEHP

The major route of exposure is *via* ingestion or inhalation (ATSDR 2002; Barrett 2006). However, patients undergoing certain medical treatments like intubation, i.v. nutrition, blood transfusion or HD are especially exposed to DEHP. Due to its lipophilic nature DEHP leaches into the blood easily.

Only a very limited amount of toxicokinetic studies in humans is available; most of the studies are performed in rodents, following oral exposure. After oral exposure of up to 200 mg/kg bw DEHP around 50% of the dose is absorbed in non-human primates (marmosets). At higher concentrations the absorption seems to be dose limited (Rhodes, Elcombe et al. 1983). If humans are exposed to DEHP *iv,* 100% is bioavailable. After absorption DEHP is rapidly metabolised in the intestine and liver to its corresponding monoester - mono(2-ethylhexyl)phthalate (MEHP) - and 2-ethylhexanol (Albro and Thomas 1973). Due to its weak polarity MEHP cannot be excreted directly. It undergoes oxidations by which secondary products like mono-[2-ethyl-5-hydroxylhexyl] phthalate (5OH-MEHP), mono-[2-ethyl-5-oxylhexyl]phthalate (5oxo-MEHP), mono-[2-ethyl-5-carboxypentyl]phthalate (5cx-MEPP), mono-[2-(carboxymethyl)hexyl]phthalate (5cx-MMHP) are formed (Fig. A-5). Both, the monoester and the oxidative metabolites can be conjugated to glucuronic acid and excreted in urine or faeces. (ATSDR 2002; Koch, Bolt et al. 2004; Koch, Bolt et al. 2005; Calafat and McKee 2006; Koch, Preuss et al. 2006). Depending on the study and species 4 – 68% (humans: 15-25%) of the DEHP is excreted by urine with a half life of 6 - 18 h (Ikeda, Sapienza et al. 1980; Peck and Albro 1982; Schmid and Schlatter 1985; Astill, Barber et al. 1986).

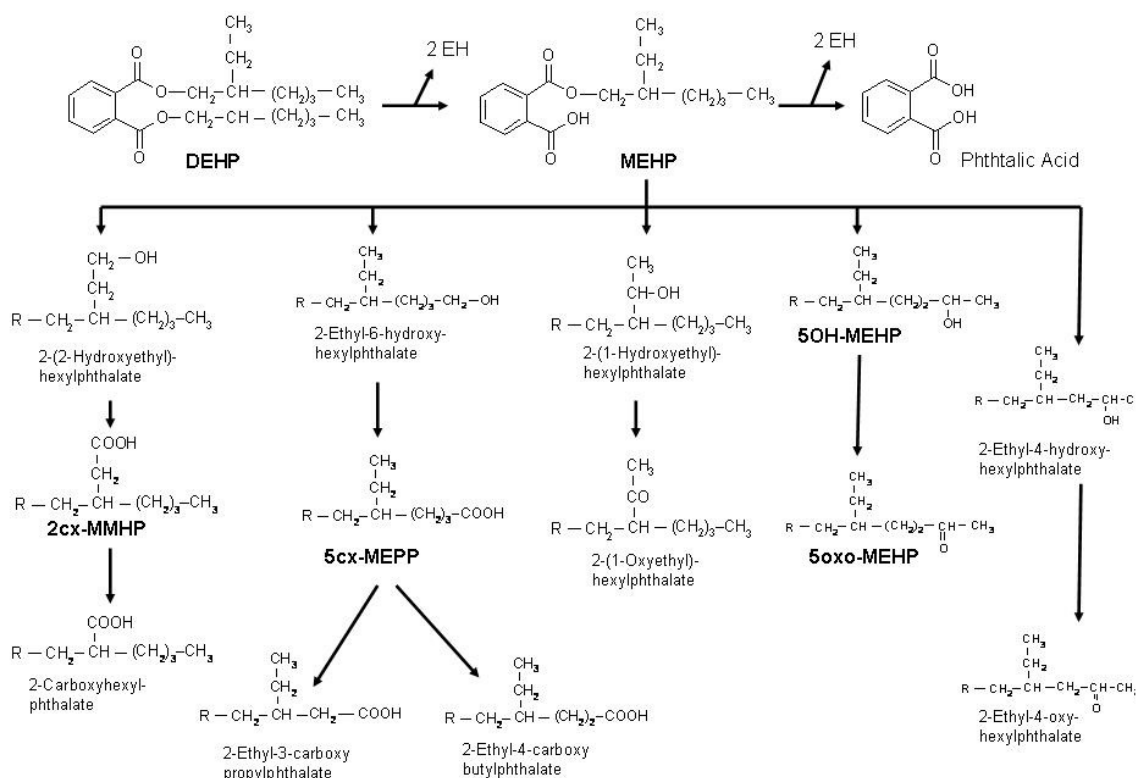


Fig. A-5 Metabolism of di(2-ethylhexyl)phthalate (adapted after Koch, Preuss et al. 2006)

In order to estimate the DEHP burden of the general population, urinary metabolites of DEHP – mostly MEHP – have been measured by various groups (Tab. A-3).

Study	n	MEHP	5OH-MEHP	5oxo-MEHP
Blount, Silva et al. 2000	289	2.7	n.a.	n.a.
Hoppin, Brock et al. 2002	46	7.3	n.a.	n.a.
Koch, Rossbach et al. 2003	85	10.3	46.8	36.5
Barr, Silva et al. 2003	62	4.5	35.9	28.3
Kato, Silva et al. 2004	176	< LOD	17.4	15.6

Tab. A-3 Median body burden of DEHP, expressed in the urinary concentration of its metabolites (in µg/ml)
n.a., not analysed; MEHP, mono(2-ethylhexyl)phthalate; 5OH-MEHP, mono-[2-ethyl-5-hydroxylhexyl] phthalate; 5oxo-MEHP, mono-[2-ethyl-5-oxylhexyl] phthalate

Based on the metabolites detected in urine, the actual DEHP intake is estimated to be ≈ 30 µg/kg bw per day (Doull, Cattley et al. 1999) or 5.6 to 21 µg/kg bw per day in adults and 7.7 to 25 µg/kg/day in children (Koch, Preuss et al. 2006).

Estimations about the additional burden of HD patients range from 3.6 to 150 mg per dialysis session (Pollack, Buchanan et al. 1985; Flaminio, Bergia et al. 1988; Faouzi, Dine et al. 1999; Dine, Luyckx et al. 2000).

2.2.3 *In vitro* and *in vivo* Effects of DEHP

2.2.3.1 Acute Toxicity

The acute oral toxicity of DEHP is very low. The LD_{50} in rat is > 20,000 mg/kg (NTP 1982) and > 9,860 mg/kg in mice (Nuodex 1981).

However, it is very likely that the metabolites, not DEHP itself, are the bioactive forms (Calafat and McKee 2006). DEHP or its metabolites produce a wide spectrum of toxic effects in multiple organ systems of laboratory animals like liver, reproductive tract, kidneys, lungs and heart (reviewed in Tickner, Schettler et al. 2001; Bureau 2003). However, these symptoms did only develop at a very high dosages (> 100 mg/kg bw to several g/kg bw), which are not relevant for the *in vivo* situation.

However, a lot of concern has been raised about possible toxicity at lower doses especially reproductive toxicity and mutagenic and hepato-carcinogenic effects in laboratory animals.

2.2.3.2 Mutagenicity

The possible genotoxic effects of DEHP and its metabolites have been thoroughly investigated. DEHP and its metabolites were tested negative in Ames tests of several *Salmonella typhimurium* strains with and without metabolic activation (Zeiger, Haworth et al. 1982; Kirby, Pizzarello et al. 1983; Yoshikawa, Tanaka et al. 1983; Zeiger, Haworth et al. 1985; Schmezer, Pool et al. 1988; Dirven, Theuws et al. 1991).

DEHP and its metabolites were also non-mutagenic in mouse lymphoma L5178Y mutation tests (Kirby, Pizzarello et al. 1983; Astill, Barber et al. 1986), did not induce DNA damage, chromosomal aberrations or sister chromatide exchange in Chinese hamster ovary cells (Phillips, James et al. 1982; Douglas, Hugenholtz et al. 1986), and did not induce DNA damage or repair in mouse, rat or human hepatocytes (Butterworth, Bermudez et al. 1984; Smith-Oliver and Butterworth 1987). They also

tested negative in micronucleus assays (Astill, Barber et al. 1986; Douglas, Hugenholtz et al. 1986). However, a few studies report induction of cell transformation in Syrian hamster embryo cells (Sanner, Mikalsen et al. 1991; Mikalsen and Sanner 1993; Tsutsui, Watanabe et al. 1993) and DNA damage detectable by comet-assay in human lymphocytes (Anderson, Yu et al. 1999). Most *in vivo* studies for DNA damage/repair and DNA adduct formation in DEHP feeding studies were negative up to 1000 mg/kg bw day (Butterworth, Bermudez et al. 1984; Kornbrust, Barfknecht et al. 1984; Gupta, Goel et al. 1985; Smith-Oliver and Butterworth 1987). Only one study reported increased 8-OH-dG levels (2-fold) in rat liver after one month DEHP containing diet (Takagi, Sai et al. 1990); another study reported a 5-fold increase in single strand breaks, but only in tumour-bearing rats (Tamura, Iida et al. 1991). They concluded that this effect is not due to direct genotoxicity. With regard to these results the US EPA and EU Commission classified DEHP as not mutagenic (Doull, Cattley et al. 1999; Bureau 2003).

2.2.3.3 Carcinogenicity

Even though DEHP and its metabolites are not genotoxic, several feeding studies conclude that it is hepato-carcinogenicity in rodents. A long-term feeding study of the US National Toxicology Program showed that DEHP induced hepatocellular carcinomas in F344 rats and B6C3F1 mice in a dose-dependent manner (Kluwe, Haseman et al. 1982; NTP 1982). These results were confirmed by other groups (Cattley, Conway et al. 1987; Popp, Garvey et al. 1987; Rao, Usuda et al. 1987; David, Moore et al. 1999). The lowest observed adverse effect level (LOAEL) derived from those studies for rats is 147 mg/kg bw per day and for mice 292 mg/kg bw per day DEHP in the diet.

The mechanism through which DEHP induces liver tumours in rodents is probably by peroxisome proliferation. Peroxisomes are cytoplasmic organelles which contain a number of hydrogen peroxide generating oxidases, catalases and fatty acid β-oxidation enzymes (Reddy 2004).

The ability of DEHP to act as peroxisome proliferator is due to its metabolite MEHP. MEHP interacts with the peroxisome proliferator–activated receptor α (Ppar-α), thereby increasing the size and the number of peroxisomes *in vivo* (Moody and Reddy 1978.; Rao and Reddy 1991; Reddy 2004). Ppar-α activation also leads to changes in gene expression, e.g. increased β-oxidation and ω-oxidation.

Subsequently more H_2O_2 is generated, which increases the oxidative stress and free radical production, thereby causing DNA damage (Doull, Cattley et al. 1999). It is also assumed that peroxisome proliferators increase cell proliferation and inhibit apoptosis, which may also contribute to cancer development (Tickner, Schettler et al. 2001).

However, relevance of theses studies for humans is highly unlikely, because humans express far less Ppar-α (1-10% of the level found in rodents (Palmer, Hsu et al. 1998)). There are also genetic variations of human Ppar-α, which render it less active compared to the rodent form (Palmer, Hsu et al. 1998; Woodyatt, Lambe et al. 1999). This is in line with the observation in large numbers of patients which are treated with peroxisome proliferating drugs (e.g. hypolipidemic drugs), who show no increased cancer incidence (Doull, Cattley et al. 1999). Additionally, studies with Ppar-α (-/-) mice found no hepatic carcinomas after DEHP administration (Peters, Cattley et al. 1997). However, these mice exhibited the remaining adverse effects attributed to DEHP: testicular lesion, kidney effects and fetotoxicity (Peters, Taubeneck et al. 1997; Ward, Peters et al. 1998).

2.2.3.4 Reproductive and Developmental Toxicity

There have been multitudes of studies analysing the developmental and reproductive toxicity of DEHP and its metabolites. As an extensive discussion of all studies would be beyond the scope of this thesis, only a relevant subset is discussed below. The additional studies are compiled in the risk assessment report on DEHP by the European Chemical Bureau (ECB) (Bureau 2003) and the risk assessments by the US Agency for Toxic Substances and Disease Registry (ATSDR) and Environmental Protection Agency (EPA) (Kavlock, Barr et al. 2006).

DEHP affects fertility and reproduction of both sexes; it also interferes with the development of the offspring. While other phthalates also possess some estrogenic activity, the reproductive toxicity of DEHP is the highest (Heindel, Gulati et al. 1989). Repeated exposure to DEHP induced testicular toxicity in male rats and mice. The effects included: reduced testis weight, reduced sperm production, reduced testosterone production, seminiferus tubular atostrophy, vacuolisation of Sertoli cells and undescended testis (Gray and Butterworth 1980; Shiota and Nishimura 1982; Lamb, Chapin et al. 1987; Tyl, Price et al. 1988; Poon, Lecavalier et al. 1997; Gray, Ostby et al. 2000; Moore, Rudy et al. 2001; Wolfe 2003; Andrade, Grande et al.

2006; Dalsenter, Santana et al. 2006). Non-human primates seem to be less sensitive towards DEHP toxicity. Exposure of up to 2500 mg/kg bw per day did not influence the testicular development in male marmosets (Tomonari, Kurata et al. 2006).

These toxic effects were especially severe when animals were exposed *in utero* and/or before they were sexually mature. The lowest reported LOEL for developmental toxicology for *in utero* exposure and during suckling are as low as 3.5 mg/kg bw per day (Arcadi, Costa et al. 1998). The current NOELs which are selected for the human risk characterisation are the 4.8 mg/kg bw per day (Wolfe 2003) or 3.7 mg/kg bw per day (Poon, Lecavalier et al. 1997).

The main targets of DEHP - or rather its metabolite MEHP - in male animals are the Leydig and Sertoli cells. Sertoli cells provide support for the germ cells and respond to follicle stimulating hormone (FSH). FSH is necessary for the initiation and maintenance of spermatogenesis. The main targets in female animals are the granulosa cells (the equivalent of Sertoli cells in males). MEHP prevents the FSH stimulation of granulosa cells *in vitro.* This leads to decreased estradiol production, prevents ovulation and prolongs the estrous cycle (Reviewed in Lovekamp-Swan and Davis 2003).

This hormone pathway is analogous in humans and rodents. It is therefore reasonable to assume that DEHP does also affect humans. Limited studies on the effect of DEHP on human populations suggest that occupational exposure to DEHP *via* PVC increases the risk for testicular cancer (Hardell, Ohlson et al. 1997). Higher levels of phthalates in human serum are also correlated with increased pregnancy complications, decreased pregnancy rates, endometriosis (Cobellis, Latini et al. 2003), short angogenital distance in male offspring (Swan, Main et al. 2005) and abnormal reproductive development (Colon, Caro et al. 2000).

2.2.4 Concerns

At the moment there is a controversial debate about the danger of phthalates as endocrine disruptors in humans (Lottrup, Andersson et al. 2006; Marsee, Woodruff et al. 2006; McEwen and Renner 2006; Queiroz and Waissmann 2006). In order to limit the risks DEHP has been banned from use in children’s toys and personal care products (e.g. cosmetics, lotions, perfumes) within the EU since 2005

(Umweltbundesamt 2006). The discussion weather DEHP should be or can be replaced in medical devices is still ongoing.

3 Uremic Toxins

As renal failure progresses and renal clearance declines, compounds which are normally excreted begin to accumulate in the blood. A number of these retention solutes exhibit toxic properties. Additionally they can become substrates for further biological reactions in the uremic milieu and can thereby contribute to the adverse effect (Himmelfarb, Stenvinkel et al. 2002). These substances are called uremic toxins.

A uremic toxin has to meet the following criteria:

- it is a chemical or biological agent capable of producing a response
- it interacts with the biological system and produces a biological response
- the response should be considered deleterious to the biological system (Vanholder, Argiles et al. 2001)

At the moment more than 90 uremic toxins are known but the number is increasing constantly (Tab. A-4) (Vanholder, De Smet et al. 2003). The uremic toxins differ in their molecular weight and their binding capacity to proteins. There are free water soluble low molecular-weight solutes like urea [MG < 500 Da]; protein bound toxins like homocysteine (Hcy) and advanced glycation end-products (AGEs) [MW mostly < 500 Da]; and middle molecules like tnf-α or leptin [MW 500 - 15,000 Da] (Vanholder, De Smet et al. 2003).

Some of the uremic toxins which are suspected to possess genotoxic capacity are discussed below.

Uremic toxins:
Free water-soluble low-MW solutes e.g.:

- Creatinine
- Cytidine
- Mannitol
- Methylguanidine
- Oxalate
- Urea.....
- …

Protein-bound solutes e.g.:

- Homocysteine
- Indoxyl sulfate
- Indole-3-acetic acid
- Leptin
- Methylglyoxal (AGE)
- …

Middle molecules e.g.:

- β2-microglobulin
- Interleukin-6
- Neuropeptide Y
- Tumor necrosis factor-α
- …

Tab. A-4 Examples of uremic toxins (Vanholder, De Smet et al. 2003)

3.1 Homocysteine

3.1.1 Chemical Structure and Pathways

Homocysteine (Hcy; 2-amino-4-mercaptobutyric acid) is an analogue of the essential, sulphur-containing amino acid cysteine (Fig. A-6).

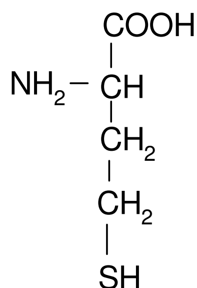


Fig. A-6 The chemical structure of homocysteine

It can be metabolised by two pathways: the transsulfuration sequence or the re-methylation cycle (Finkelstein 1990) (Fig. A-7). The re-methylation cycle outweighs

the transsulfuration sequence in most mammalian cells. In this pathway Hcy is re-methylated to methionine (Met) by the vitamin-B_{12} dependent methylfolate-homocysteine methyltrasferase, using 5-methyltetrahydrofolate as methyl donor. The generated Met is converted to S-adenosylmethionine (SAM) – a methyl donor for DNA and proteins. SAM takes part in numerous specific transmethylation reactions which yield S-adenosylhomocysteine (SAH) as a product. Finally the adenosyl-homocysteinase uses SAH to synthesize Hcy (Finkelstein and Martin 2000).

Apart from this basic re-methylation cycle, Hcy can also be methylated by a second Hcy-methylase, which employs betaine as methyl donor. This Hcy-methylase is primarily found in the liver of all mammalian species and in the primate kidney (McKeever, Weir et al. 1991).

The transsulfuration pathway leads to the irreversible removal of Hcy from the organism. At first, the cystationine-β-synthetase (vitamin B_6-dependent) catalyses the reaction of Hcy with serin. In this process cystathione is formed. Ammoniac and α-ketobtyrate are cleaved from cystathione by the cystathionine-γ-lyase forming cysteine (Lehninger, Nelson et al. 1994). Cysteine is an important precursor for the synthesis of the cellular antioxidant glutathione. The transsulfuration pathway is found primarily in the liver, kidney, pancreas and intestine (Finkelstein 1990). Apart from participating in these two pathways, Hcy can also form the reactive compound homocysteine-thiolactone (Hcy-T) (s. 3.1.6, page 27).

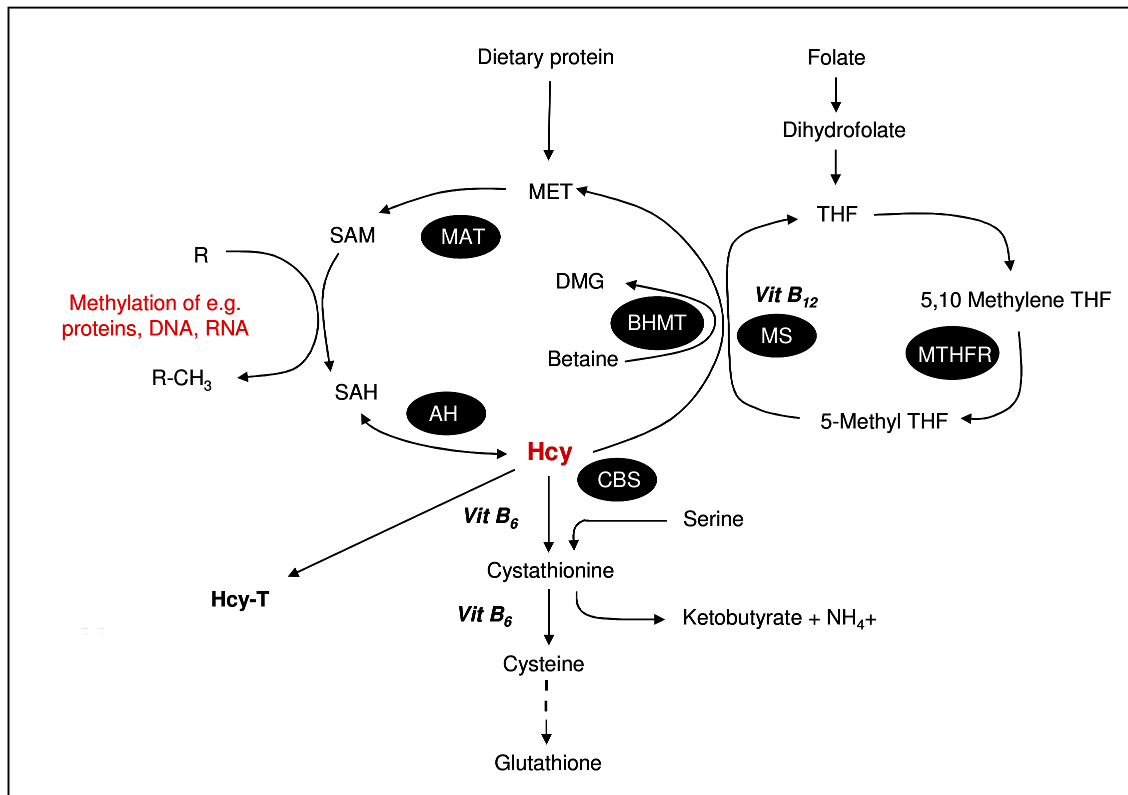


Fig. A-7 Hcy metabolism and the enzymes and vitamins involved
Hcy is an intermediate in the sulfur amino acid metabolism. It is linked with the methionine cycle (left) and the folate cycle (right). It can finally be removed from these cyles *via* the transsulfuration pathway.
Abbreviations: AH, adenasyl-homocysteinase; BHMT, betaine-homocysteine-S-methyltransferase; CBS, cystathione-β-synthase; Hcy, homocysteine; Hcy-T, homocysteine-thiolactone; MAT, methionine-adenosyltransferase; MET, methionine; MS, methionine synthase; MTHFR, methylenetetrahydrofolate reductase; SAH, S-adenosylhomocysteine; SAM, S-adenosylmethionine; THF, tetrahydrofolate

Intracellularly, Hcy is present in its free and reduced forms but the concentration remains within strict limits. The main regulatory mechanism for maintaining the Hcy equilibrium in the cell is *via* export into the plasma (Reviewed in Blom and De Vriese 2002).

After entering the bloodstream Hcy is rapidly oxidized. Seventy percent are bound to proteins mainly serum albumin the rest occurs as homocystin - the disulfide of homocysteine - and homocysteine-cysteine mixed disulfides, while only 1 - 2% exist as free, reduced thiol (Finkelstein and Martin 2000).

3.1.2 Homocysteine Levels

The normal plasma concentration of total homocysteine is between 8 – 10 µM in females and 10 - 12 µM in males (Perna, Ingrosso et al. 2003). Mild (12 - 15 µM) to moderate hyperhomocysteinemia (16 - 30 µM) is found in 5 - 10% of the population (Stanger, Herrmann et al. 2003). Values between 31 – 100 µM are regarded as intermediate hyperhomocysteinemia and values > 100 µM as severe hyperhomocysteinemia (Perna, Ingrosso et al. 2004). The plasma levels of Hcy increase with age (Hernanz, Fernandez-Vivancos et al. 2000) and are generally higher in men than in women (Lussier-Cacan, Xhignesse et al. 1996). This is probably an estrogenic effect as the gender difference disappears after menopause. The intra-individual variability is very low and no seasonal effect could be determined.

One group of persons in which hyperhomocysteinemia is very common are patients suffering from ESRD (Suliman, Qureshi et al. 2000; Mallamaci, Zoccali et al. 2002; Kalantar-Zadeh, Block et al. 2004; Wrone, Hornberger et al. 2004; Nair, Nemirovsky et al. 2005; Perna, Satta et al. 2006). Roughly 90% of these patients suffer from hyperhomocysteinemia, mostly moderate to intermediate hyperhomocysteinemia, although severe cases are not uncommon.

The plasma Hcy level strongly correlates with the glomerula filtration rate, but the precise mechanism is not definitely established (van Guldener 2006). Unlike in many healthy, but folate deficient persons, the administration of folic or folinic acid does not normalize the Hcy levels in HD patients (Armada, Perez et al. 2003). However, it still reduces the Hcy level.

3.1.3 Reasons for Elevated Homocysteine Levels

The reasons for hyperhomocystemia in ESRD patients are complex and still not completely understood. Impaired renal excretion of Hcy was thought to be the underlying reason, but as the amount of Hcy in the urine is only about 6 µmol/l this seems unlikely (Refsum, Helland et al. 1985). Another hypothesis is that uremic toxins impair some of the enzymes, relevant to the Hcy metabolism (van Guldener and Stehouwer 2003; Perna, Ingrosso et al. 2004). This is in line with the observation of a decreased remethylation and transmethylation flux in ESRD patients, while the transsulfuration rate of those patients is similar to control subjects (van Guldener, Kulik et al. 1999; van Guldener 2006). Furthermore, uremic patients, with their poor appetites and recommended low protein diets, are under constant duress to produce

enough methyl groups to sustain the normal transmethylation rate they need to prevent the accumulation of Hcy. The same holds true for the vitamin supply of cells, especially as the transmembrane transport of folate is impaired in ESRD patients (Jennette and Goldman 1975).

Apart from ESRD, gene mutations, reduced vitamin intake or intestinal absorption as well as the intake of certain drugs can also lead to increased Hcy levels (Tab. A-5).

Causes/determinants
Genetic factors:

- Homocystinuria
- Heterozygosity for CBS defects
- Down syndrome
- MTHFR 677C→T (homozygosity)
- Other polymorphisms

Physiologic determinants:

- Increasing age
- Male sex
- Pregnancy
- Postmenopausal state
- Renal function, reduced GFR
- Increasing muscle mass

Lifestyle determinants:

- Vitamin intake (folate, B12, B6, B2)
- Smoking
- Coffee
- Ethanol
- Exercise

Clinical condition:

- Folate deficiency
- Cobalamin deficiency
- Vitamin B6 deficiency
- Renal failure
- Hyperproliferative disorders
- Hypothyroidism
- Hyperthyroidism
- Diabetes

Drugs:

- Lipid lowering (cholestyramine, fibric acid derivates, nicotinic acid)
- Anticonvulsants (phenytoin, carbamazepine)
- Sex hormones (androgens)
- Anti-rheumatic drugs (methotrexate)
- Other (cyclosporin, diuretics, levodopana)

Tab. A-5 Determinants of plasma total Hcy (Hankey, Eikelboom et al. 2004)

3.1.4 Clinical Implications of Elevated Homocysteine Levels

The first researcher to suggest adverse effects of Hcy was McCully in 1969. He proposed that elevated levels of Hcy cause arteriosclerosis (McCully 1969). Since then, elevated levels of total Hcy have also been related to birth defects and

pregnancy complications (Miller and Kelly 1996; Vollset, Refsum et al. 2000) as well as psychiatric disorders (Nilsson, Gustafson et al. 1996) and cognitive impairment in the elderly (Dimopoulos, Piperi et al. 2006; McMahon, Green et al. 2006).

Most importantly, increased plasma concentrations of total Hcy have been regarded as strong and independent risk factors for cardiovascular disease and stroke (Nygard, Nordrehaug et al. 1997; Vollset, Refsum et al. 2001; Collaboration 2002; Wald, Law et al. 2002). However, within the last few years three large and well-conducted prospective studies have set off a controversy whether total Hcy is a risk factor or merely an innocent bystander of the disease (Bonaa, Tverdal et al. 2006; Craen, Stott et al. 2006; Khare, Lopez et al. 2006; Lonn 2006; Quinlivan and Gregory 2006; Refsum and Smith 2006): the Heart Outcome Prevention Evaluation 2 (HOPE-2) (Lonn, Yusuf et al. 2006), the Vitamin Intervention for Stroke Prevention trial (VISP) (Toole, Malinow et al. 2004) and the Norwegian Vitamin trials (NORVIT) (Bonaa, Njolstad et al. 2006). All studies included several thousand participants (HOPE-2: 5522, VISP: 3680 and NORVIT: 3749) and showed that lowering of tHcy by administration of B vitamins and/or folate did not reduce the risk of cardiovascular events or stroke compared to a placebo group. However, these studies covered only a two to five year period, therefore one cannot rule out that a positive effect might be observed at a later date.

Conflicting results have been reported for total Hcy and mortality in ESRD patients. While some studies report a strong association between hyperhomocysteinemia and cardiovascular mortality (Bostom, Shemin et al. 1997; Moustapha, Naso et al. 1998; Mallamaci, Zoccali et al. 2002), others could not confirm this association (Menon, Sarnak et al. 2006) or even reported an inverse relationship (Suliman, Qureshi et al. 2000; Kalantar-Zadeh, Block et al. 2004; Wrone, Hornberger et al. 2004; Nair, Nemirovsky et al. 2005; Ducloux, Klein et al. 2006).

Additionally, high total Hcy levels have even been discussed as new tumour marker (Wu and Wu 2002)

3.1.5 Hyperhomocysteinemia and Cancer

Several studies have found a significant positive correlation between elevated levels of total Hcy and increased MN frequency in healthy men between 50 and 70 (Fenech, Dreosti et al. 1997; Fenech 1999). This observation was also true for young Australian males [18 - 32 years], but not females (Fenech, Aitken et al. 1998). These

correlations are supported by preliminary *in vitro* studies on human lymphocytes. There was a moderate increase of MN in cells treated with 50 – 400 µM Hcy (Crott and Fenech 2001).

Further evidence for the involvement of Hcy in cancer development is its influence on DNA cytosine-methylation; however the results of these studies are conflicting. One study on patients with chronic alcoholism correlated Hcy levels with DNA hypermethylation in peripheral blood cells of patients (Bonsch, Lenz et al. 2004), while a small study on ESRD patients found that plasma total Hcy concentration correlated significantly with DNA hypomethylation (Ingrosso, Cimmino et al. 2003).

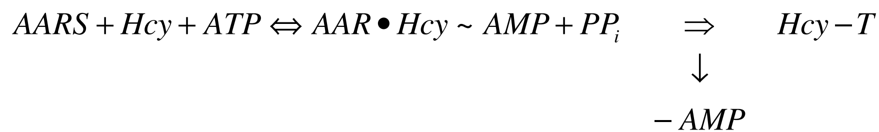
Oxidative stress may also lead to DNA damage. Although all dialysis patients suffer from increased oxidative stress, the discussed whether Hcy contributes is still ongoing (Bayes, Pastor et al. 2001; Bayes, Pastor et al. 2003).

The same holds true for *in vitro* tests; while Hcy elicited oxidative stress in some test systems (Austin, Sood et al. 1998; Au-Yeung, Woo et al. 2004; Perez-de-Arce, Foncea et al. 2005), it did not in others (Outinen, Sood et al. 1998; Lynch, Campione et al. 2000; Zappacosta, Mordente et al. 2001) or had antioxidant as well as pro-oxidant effects (Lynch and Frei 1997).

3.1.6 Homocysteine-Thiolactone

Homocysteine-thiolactone (Hcy-T) is formed in human cells by the enzymatic conversion of Hcy to its corresponding thioester. This conversion occurs when Hcy falsely enters the protein biosynthetic apparatus because of its similarity to methionine, isoleucin and leucin. However, Hcy cannot complete the biosynthetic pathway (Jakubowski 2004).

After misactivation by methionyl-tRNA to Hcy-AMP (thereby using ATP), Hcy-AMP is subsequently destroyed by the enzyme forming a cyclic thioester - Hcy-T (Fig. A-8). This reaction is universal to all cells and prevents the misincorporation of Hcy into proteins (Jakubowski 1997). Hcy-T can be reconverted to Hcy by enzymes like the intracellular bleomycin hydrolase or the extracellular HDL-associated - Hcy-thiolactonase (Jakubowski 2006).



$$AARS + Hcy + ATP \Leftrightarrow AAR \bullet Hcy \sim AMP + PP_i \quad \Rightarrow \quad Hcy - T$$
$$\downarrow$$
$$-AMP$$

Fig. A-8 Equation for the formation of Hcy-T (Jakubowski 1999)
AARS: aminoacyl-tRNA; Hcy: homocysteine; ATP: adenosine triphosphate; AMP: adenosine monophosphate; pp_i: pyrophosphate; Hcy-T: homocysteine-thiolactone

The energy of the anhydride bond Hcy~AMP is conserved in an intra-molecular thioester bond of Hcy-T. Consequently, Hcy-T is chemically reactive and acetylates free aminogroups easily (Jakubowski 1999).

Under physiological conditions Hcy-T is neutral in charge and freely diffuses through cell membranes (Jakubowski and Goldman 1993; Jakubowski 1997) (Fig. A-9). Therefore it is assumed that increasing levels of Hcy lead to increasing misactivation, finally leading to the formation of Hcy-T, which then leaves the cell.

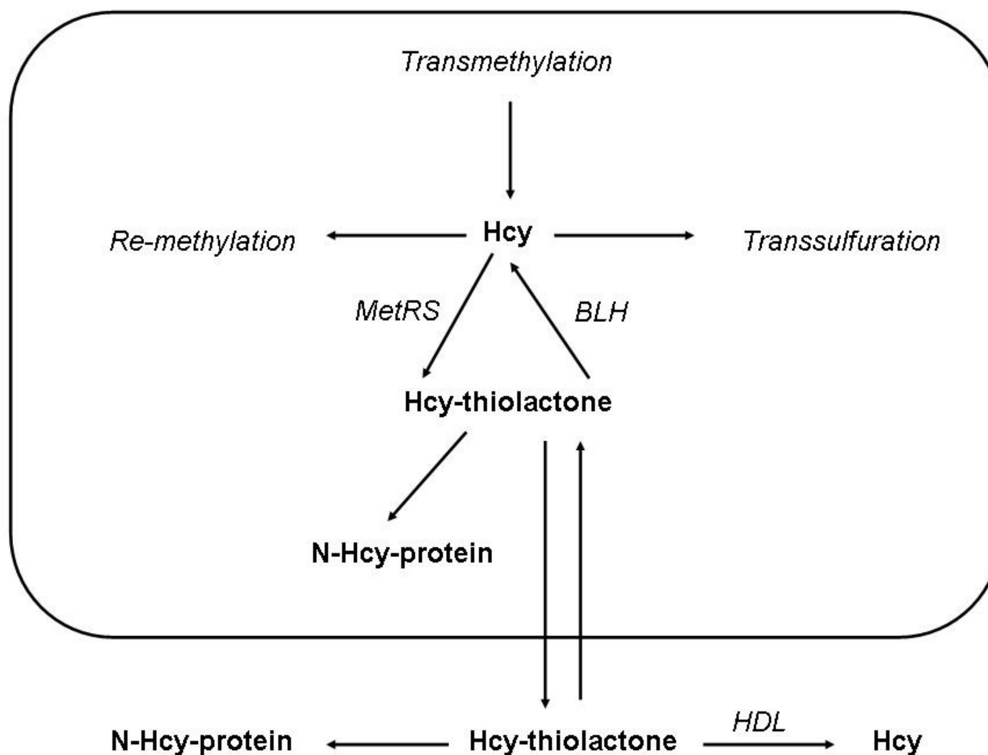


Fig. A-9 Hcy/Hcy-T metabolism (adapted after Jakubowski 2006)
MetRS, methionyl-tRNA synthetase; BLH, bleomycin hydrolase; HDL, HDL-associated -Hcy-thiolactonase

In plasma Hcy-T undergoes two major reactions: (1.) acylation of aminogroups in proteins (primarily ε-amino groups of lysine residues) and (2) enzymatic hydrolysis to give Hcy, which then attaches to proteins by forming a protein-S-S-Hcy disulfide (Jakubowski 1999). This may lead to protein damage.

The chemical reactivity of Hcy-T leads to a half-life of approximately 1 h in blood and plasma (Jakubowski 1999). In healthy subjects the Hcy-T levels vary between 0 – 34.8 nM (Daneshvar, Yazdanpanah et al. 2003; Chwatko and Jakubowski 2005); representing about 0 – 0.28% of tHcy. Surprisingly, there is no correlation between total Hcy levels and Hcy-T concentration in plasma (Jakubowski 2006). This suggests that it is not Hcy which is the major determinant of Hcy-T but HDL-associated-Hcy-thiolactonase, methionyl-tRNA synthetase or renal excretion. In fact, Hcy-T is efficiently eliminated by urinary excretion, with a Hcy-T concentration up to 100-fold higher than in plasma (Chwatko and Jakubowski 2005).

3.2 Advanced Glycation End-Products (AGEs)

3.2.1 Formation of Advanced Glycation End-Products

AGEs are a heterogeneous group of molecules. Six AGEs (fructoselysine, carboxymethyllysine, pyrraline, pentosidine, glyoxal-lysine dimer, methylglyoxxal-lysine-dimer) are classified uremic toxins (Vanholder, Argiles et al. 2001; Vanholder, De Smet et al. 2003). They are generated by the non-enzymatic reaction of reducing sugars and the free amino groups of individual amino acids, peptides or proteins. The unstable Schiff's base which is formed in this process can isomerise and form the Amadori product. A subsequent series of complex biochemical reactions like dehydration, condensation, fragmentation and oxidation slowly leads from Amadori product to final AGEs (Fig. A-10), (Reviewed in Bohlender, Franke et al. 2005; Sebekova, Wagner et al. 2007).

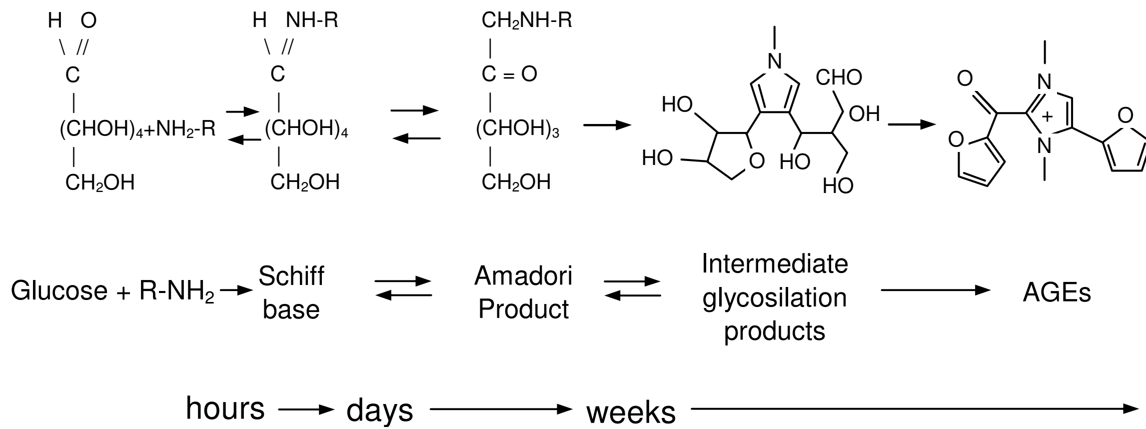


Fig. A-10 Formation of AGEs (adapted after Raj, Choudhury et al. 2000)

This process occurs endogenously or during food processing (e.g. heating). Actually, diet-derived and orally absorbed AGEs are suspected to contribute significantly to the overall AGE load in renal failure patients (Koschinsky, He et al. 1997; Uribarri, Peppa et al. 2003; Goldberg, Cai et al. 2004). Independent of their origin, AGEs are cleared by the kidney (Gugliucci and Bendayan 1996). Miyata et al. showed the fate of an exemplary AGE – pentosidine: it is filtered by the glomeruli and reabsorbed by the proximal tubule cells. There it is modified, degraded and eventually excreted in the urine (Miyata, Ueda et al. 1998). Therefore AGE levels correlate inversely with creatinine clearance, which leads to up to 6-fold elevated AGE levels in ESRD patients (Makita, Radoff et al. 1991; Makita, Bucala et al. 1994). Apart from reduced renal function, elevated plasma glucose may contribute to elevated AGE levels (Makita, Radoff et al. 1991; Brownlee 1995).

3.2.2 Biological Effects of AGEs

Elevated levels of AGEs have been correlated to several diseases, e.g. Alzheimer's disease (Gasic-Milenkovic, Loske et al. 2003; Lue, Yan et al. 2005; de Arriba, Stuchbury et al. 2007), arteriosclerosis (Turk, Sesto et al. 2003), and have been related to the aging process (Brownlee 1995) and nephropathy.

The mechanisms by which AGEs induce pathological changes are still a focus of ongoing research. One pathway is by interaction with the receptor for advanced glycation end products (RAGE). RAGE is a cell surface receptor which belongs to the immunoglobulin superfamily. Binding to this receptor leads to initiation of intracellular

signalling cascades, including NF-κB activation resulting in inflammation and immune response. This includes macrophage activation, increased cytokines, chemokines, growth factors and ROS expression (Yan, Schmidt et al. 1994; Wendt, Tanji et al. 2003 reviewed in Schmidt, Yan et al. 2001; Lin 2006). As the RAGE expression is upregulated under high AGE conditions these reactions enhances themselves (Hou, Ren et al. 2004).

Additionally AGEs have been shown to exert genotoxic effects *in vitro* (Bucala, Model et al. 1985; Mullokandov, Franklin et al. 1994; Murata, Mizutani et al. 2003; Roberts, Wondrak et al. 2003; Schupp, Schinzel et al. 2005) and may therefore be involved in the cancer development of ESRD patients.

3.3 Leptin

Leptin, the16 kDa product of the *ob* gene (obese gene), was discovered in 1994 by Zhang et al. (Zhang, Proenca et al. 1994). The 167 amino acid hormone is mainly produced by adipocytes and has gained interest because of its involvement in the regulation of food intake and energy expenditure. Today it is known that leptin acts as a multifunctional hormone (Fruhbeck 2006) and influences the immune system (Lam and Lu 2007), female and male reproduction, the mammary glands, the gut, the kidney and the lungs (Baratta 2002) as well as blood pressure (Fruhbeck 1999), bone formation and angiogenesis.

Leptin exerts its physiological function through the binding to Ob-receptors, which belong to the cytokine class 1. To date, six isoformes of the leptin receptor are known, which are generated by alternative splicing of the *db* gene (Harvey 2007). Those receptors have identical extracellular and transmembrane domains but differ in their intracellular domain. The Ob-Rb is the most important receptor, because it is the only one which is fully functional and can submit a signal *via* the JAK/STAT (Janus kinases/signal transducers and activators of transcription) or MAPK (mitogen-activated protein kinase) pathways. The main function of the other receptors is probably transport of leptin through the brain barrier and uptake of leptin for degradation (Huang and Li 2000; Chelikani, Glimm et al. 2003; Hegyi, Fulop et al. 2004).

Plasma levels of leptin vary between 11.9 ± 3.1 µg/l (males) and 21.2 ± 3 µg/l (females), being higher in females than in males, and are closely related to the amount of body fat and BMI (Horn, Geldszus et al. 1996).

3.3.1 Leptin – an Uremic Toxin?

In many (Merabet, Dagogo-Jack et al. 1997; Sharma, Considine et al. 1997) but not all (Stenvinkel, Heimburger et al. 1997) ESRD patients, leptin levels are enhanced by the 2 - 4 fold. However, only the level of free leptin in the plasma is increased, while the protein bound fraction remains stable (Widjaja, Kielstein et al. 2000). In spite of the elevated leptin levels, ESRD patients have significantly lower leptin mRNA expression compared to BMI matched controls. This is possibly due to a negative feedback regulation by decreased renal clearance (Nordfors, Lonnqvist et al. 1998).

Due to the multifunctional nature of leptin, it is easy to imagine that elevated levels may have a negative impact on the patient. Still, leptin is only classified as suspected uremic toxin (Vanholder, De Smet et al. 2003). However, the evidence for negative impact of leptin in dialysis patients is increasing. Because leptin reduces appetite and increases the metabolic rate, it has been speculated whether leptin might be one of the factors that mediates anorexia and wasting syndrome in ESRD patients. So far the study results are controversial: some fail to find a correlation between nutritional markers and hyperleptinemia (Koo, Pak et al. 1999; Rodriguez-Carmona, Perez Fontan et al. 2000), while others find an inverse correlation (Young, Woodrow et al. 1997; Johansen, Mulligan et al. 1998).

It has been shown that leptin stimulates proliferation and differentiation of hemopoietic cells (Gainsford, Willson et al. 1996), and acts as an act anti-apoptotic (Konopleva, Mikhail et al. 1999, Artwohl, 2002) thereby promoting the proliferation of colorectal cancer cells(Rouet-Benzineb, Aparicio et al. 2004; Ogunwobi and Beales 2007). It also promoted the proliferation of prostate cells (Deo, Rao et al. 2008). In support of these *in vitro* findings, leptin levels of HD patients were correlated with peripheral DNA damage detectable by comet assay (Horoz, Bolukbas et al. 2006).

4 Cancer

Cancer is a complex disease in which altered gene expression leads to abnormal cell proliferation and invasion of other tissues, thereby disrupting their normal function. There are several theories about the cause of cancer but all of them assume that several critical mutations have to take place until a cancer phenotypic cell is formed (Fenech 2002). Critical mutations are located e.g. within apoptosis

genes, oncogenes, tumorsuppressor genes, mismatch repair genes or cell cycle control genes.

Given that an accumulation of mutations cannot be explained by single point mutations, it has been suggested that mutations lead to hypermutatbility, which later initiates cancer (Cahill, Kinzler et al. 1999; Tomlinson 2001; Coleman and Tsongalis 2006).

One possible mechanism which converts a cell that way is by generation of aneuploidy (abnormal number of chromosomes due to loss or gain of one or more chromosome) (Li, Sonik et al. 2000; Fenech 2002). According to this hypothesis the generation of cancer cells follows two steps: (1) a carcinogen leads to aneuploidy; (2) aneuploidy destabilizes the karyotype finally leading to neoplastic karyotypes (Li, Sonik et al. 2000).

Another factor which is very likely to increase the mutation frequency is direct DNA damage. Indeed, several well-documented studies correlate DNA damage and cancer in laboratory animals as well as in humans (Hagmar, Bonassi et al. 1998).

4.1 Types of DNA Damage

There are several types of DNA damage: single strand breaks, mismatch of bases, hydrolysis of bases, alkylation of DNA, DNA cross-linking, DNA oxidation and changes in DNA methylation patterns. The types relevant for the present thesis are discussed below.

4.1.1 DNA Oxidation

DNA oxidation occurs when the DNA is attacked by reactive oxygen species (ROS). ROS are produced e.g. during cellular respiration, which involves the reduction of O_2 to H_2O in the electron transport chain. Intermediates of this process are ROS like $O_2^{-\cdot}$, H_2O_2 and (HO·). If a substance interferes with the electron transport chain or disrupts the mitochondrial membrane, some of these ROS can escape the electron transport chain. Additionally, several oxidizing enzymes like e.g. NADPH-oxidase, as well as toxins or radiation can produce ROS.

Under normal circumstances, ROS are converted to water and molecular oxygen by the enzymatic antioxidant defence system of the cell. However, if there is an imbalance between antioxidants and ROS (oxidative stress), ROS - especially hydroxylradicals - can attack the nitrogenous bases or the sugar-phosphate-

backbone of the DNA. This leads to hydroxylation, ring opening and fragmentation (Sies 1997; Kelly, Havrilla et al. 1998; Griendling, Sorescu et al. 2000; Vaziri 2004). Normally this is repaired but if the damage is not recognised or severe this can lead to mutations.

Therefore it is not surprising that numerous studies link oxidative stress to cancer (Bendesky, Michel et al. 2006; Beevi, Rasheed et al. 2007; Hori, Oda et al. 2007; Nayak and Pinto 2007). Additionally, ROS can also react with proteins, lipids and carbonhydrates, thereby causing inflammation, apoptosis, fibrosis and cell proliferation.

4.1.2 Changes in DNA Cytosine-Methylation

DNA methylation is the epigentic modification of DNA by the covalent addition of a methyl group on the 5' carbon of cytosine within the context of the CpG nucleotide. DNA methylation patterns are established by at least three independent methyltransferases. Approximately 70% of the DNA is methylated (Robertson and Jones 2000). However, methylation patterns are not random but heritable, tissue- and species specific (Kim 2004). DNA methylation is an important factor for gene expression, DNA conformation and stability, binding of transcription factors, x-chromosome inactivation and, imprinting and suppression of parasitic DNA sequences (Kim 1999; Robertson and Jones 2000). In this process methylated DNA sequences become transcriptionally silenced (Robertson and Jones 2000).

Due to their big impact on DNA it is not remarkable that changes in DNA methylation patterns have been associated with several cancers, e.g. breast cancer (Widschwendter and Jones 2002), lung cancer (Piyathilake, Frost et al. 2001), colorectal cancer (Kim 2004) and kidney cancer (Cairns 2004). Generally, the cancer genome can be characterized by hypermethylation of specific genes and a simultaneous overall decrease of 5-methyl-cytosine (Zhu and Yao 2007). The degree of overall DNA methylation decreases progressively during the stages of neoplasia from benign proliferation to invasive cancer (Fraga, Herranz et al. 2004).

B Objectives

The objective was to examine the role of toxins in hemodialysis therapy and their possible contribution to the increased genomic damage of HD patients. Two possible sources for those toxins have been considered: (1) substances leaching from the extracorporal blood circuits, and (2) uremic toxins accumulating in the blood of HD patients.

The first part of this work focused on substances leaching from dialysers and tubing. Therefore eluates of different dialysers under conditions similar to the ones during dialysis had to be produced. Next, the substances leaching from dialyser into the eluates had to be analysed by LC-MS/MS. In order to examine the contribution of those substances to the genomic damage of HD patients, cytotoxicity and genotoxicity of those eluates had to be by analysed by *in vitro* studies. As some cancers are responsive to xenoestrogens, the estrogenic activity of eluates had to be examined by E-Screen.

The second part of the work focused on the toxicity of accumulated uremic toxins. As more than 90 uremic toxins are known, a sensible selection of the uremic toxins tested had to be made. We chose Hcy because elevated Hcy levels have been correlated to increased MN frequency and because it induced MN formation *in vitro*. In order to estimate the relevance of this genomic damage to the dialysis patients, possible mechanisms for MN induction had to be analysed. Frequent reasons for DNA damage are oxidative stress, changes in DNA methylation or disturbances during mitosis. Therefore those possibilities had to be analysed. Additionally the genotoxicity of Hcy-T, leptin and AGEs was analysed.

C Materials & Methods

1 General Materials

1.1 General Technical Equipment:

The following equipment was used for the experiments (Tab. C-1).

Product	**Manufacturer**
Autoclave	Melag Autoklav 23, Berlin, Germany
Camera	Cohn-High Performance, CDD Camera, INTAS Science Imaging Instruments, Göttingen, Germany
Centrifuges	Universal / K2S, Hettich, Tuttlingen, Germany Laborfuge 400e, Heraeus, Hanau, Germany Spectrafuge 24D, Abimed, Langenfeld, Germany Universal 16R, Hettich, Tuttlingen, Germany
Coulter Counter	Coulter Z1, Coulter Electronics, GB
Cytocentrifuge	Cytospin 3, Shandon, GB
Flow cytometer	BD FACS LSR, 2 Laser, H0108, Becton Dickinson, Heidelberg, Germany
Freezing Container	NALGENE™ Cryo 1℃, Nalgene, USA, Cat. No: 5100-0001
Heating plate	Gerhardt H22 electronic, Bonn, Germany
HPLC	Agilent 1100, Agilent Technologies, Santa Clara, USA
Incubator	Type B, 5060 EK-CO_2, Heraeus, Germany
Laminar Flow	Gruppo Flow, Gelaire BH26, Flow Laboratories, Germany $Antair_{BSK}$, BSK4, Antair
Linear ion trap mass spectrometer	QTrap™, Applied Biosystems, Darmstadt, Germany
Microscopes	Fluorescence Microscope (comet assay): Nikon, Labphot 2 A/L, Nikon, Germany Fluorescence Microscope (micronuclei): Zeiss, Jena, Germany Light microscope (cell culture): Labovert, Leitz, Wetzlar, Germany
Microwave Oven	M630, Phillips, Hamburg, Germany
Peristaltic pump	Typ: BV-GE, ISMATEC Laboratoriumstechnik GmbH, Wertheim-Mondfeld, Germany
pH meter	pH 526, Multical WTW, Weilheim, Germany
Photometer	Hitachi, U-2000, Tokyo, Japan
Pipettes	Gilson, Middleton, USA Eppendorf, Hamburg, Germany
Shaker	KL2, Edmund Büchler, Germany
Sterilizer	T 6120, Heraeus, Hanau, Germany
Triple quadrupole instrument	API 3000, Applied Biosystems, Darmstadt, Germany
Vortex	Vortex Genie 2, Bender & Hobein, Zürich, Switzerland
Water Bath	GFL, Type 1012, Gesellschaft für Labortechnik GmbH, Burgwedel, Germany GFL, Type 1083, Gesellschaft für Labortechnik GmbH, Burgwedel, Germany

Tab. C-1 General technical equipment

1.2 General Materials and Chemicals

Unless otherwise stated chemicals not listed in Tab. C-2 were purchased from Sigma-Aldrich, Taufkirchen, Germany.

Product	Manufacturer/Supplier
5 ml Multitips	Multitips, sterile, Laborbedarf Hartenstein, Würzburg, Germany, Cat. No: 4003-0013
Acridine orange	Serva, Heidelberg, Germany, Cat. No: 21572
Bio-Rad Protein Assay, Dye Reagent Concentrate	Bio-Rad, Munich, Germany, Cat. No: 500-006
Bovine Serum Albumine	Sigma-Aldrich, Taufkirchen, Germany, Cat. No: A7906
Cell culture flasks	Sarstedt, Nürnbrecht, Germany
Cryo-vials	Greiner, Solingen-Wald, Germany, Cat. No: 121263
Cuvettes (10×4×45 mm)	Sarstedt, Nürnbrecht, Germany, Cat. No: 67.704
Dialysis tubing membranes	Sigma-Aldrich, Taufkirchen, Germany
DMSO (Dimethyl sulfoxide)	Sigma-Aldrich, Taufkirchen, Germany, Cat. No: D4540
EDTA (Ethylendiamine-tetra aceticacid)	Roth, Karlsruhe, Germany, Cat. No: 8040.1
Ethanol	Roth, Karlsruhe, Germany, Cat. No: P006.1
FACS Clean	BD Biosciences, Heidelberg, Germany, Cat. No: 340345
FACS Rinse	BD Biosciences, Heidelberg, Germany, Cat. No: 340346
FACS tubes	BD Biosciences, Heidelberg, Germany, Cat. No: 343675
Falcon tubes	Sarstedt, Nürnbrecht, Germany
Methanol	AppliChem, Darmstadt, Germany, Cat. No: A0688
Methyl-Methane-sulfonate	Sigma-Aldrich, Taufkirchen, Germany, Cat. No: M4016
$NaAsO_2$	Sigma-Aldrich, Taufkirchen, Germany, Cat. No: S7400
NaCl	AppliChem, Darmstadt, Germany, Cat. No: A1149
NaOH	AppliChem, Darmstadt, Germany, Cat. No: A1551
Propidium iodide (PI)	Sigma-Aldrich, Taufenkirchen, Germany, Cat. No: P4864
Slides	Assistent-Objektträger "ELKA", Glasfabrik Karl Hecht KG, Sondheim, Germany, Cat. No: 2406
Sodium acetate	Sigma-Aldrich, Taufkirchen, Germany, Cat. No: S5636
Sterile filter Whatman®	Sigma-Aldrich, Taufkirchen, Germany, Cat. No: F8552
Super frost slides	AMNZ SuperFrost®Plus, Menzl-Gläser, Braunschweig, Germany, Cat. No: J1800
TRIS (Tris-hydroxymethyl-aminomethan)	Roth, Karlsruhe, Germany, Cat. No: A411.1
Triton X-100	Sigma-Aldrich, Taufkirchen, Germany, Cat. No: 93443
Tween 20	Sigma-Aldrich, Taufkirchen, Germany, Cat. No: P2287

Tab. C-2 General materials and chemicals

2 Cell Culture

2.1 Media, Supplements and General Buffer

Media	Manufacturer/Supplier
Dulbecco´s modified Eagle´s medium (DMEM)	Sigma-Aldrich, Taufkirchen, Germany, Cat. No: D5546
RPMI 1640 *(With $NaHCO_3$, without phenol red)*	Sigma-Aldrich, Taufkirchen, Germany, Cat. No: R7509
RPMI 1640 *(With $NaHCO_3$ and phenol red)*	Sigma-Aldrich, Taufkirchen, Germany, Cat. No: R0883
Horse serum	Biochrom AG, Berlin, Germany, Cat. No: 59135
L-glutamine (stock solution 200 mM)	Sigma-Aldrich, Taufkirchen, Germany, Cat. No: G7513
Penicillin-Streptomycine (10000 U/ml penicillin, 10mg/ml streptomycine)	Sigma-Aldrich, Taufkirchen, Germany, Cat. No: P0781
Sodium pyruvate (stock solution 100 mM)	Sigma-Aldrich, Taufkirchen, Germany, Cat. No: S8636
Accutase	Sigma-Aldrich, Taufkirchen, Germany, Cat. No: A6964
Trypsin-EDTA (stock solution: 10 x; EDTA: 2 g/l Trypsin: 5 g/l),	Sigma-Aldrich, Taufkirchen, Germany, Cat. No: T4174
Fetal Bovine Serum (FBS)	Biochrom, Berlin, Germany, Cat. No: S0115

Tab. C-3 Media and supplements

Name	Ingredients
10 × PBS (Phosphate buffered saline)	1.4 M NaCl 27 mM KCl 100 mM KH_2PO_4/K_2HPO_4 pH 7.5 diluted to 1 × PBS with ddH_2O prior to use

Tab. C-4 General buffer

2.2 Cell Lines, Media and Growth Conditions

Cell line	Cell type	Origin	Medium
HL-60	Human acute myeloid leukemia	Established from the blood of a 35-year-old female with acute myeloid leukaemia in 1976	RPMI 1640 (R0883) 10% (v/v) FBS 1% (v/v) L-glutamine 0.4% (v/v) Penicillin-Streptomycine
MCF-7	Human breast adenocarcinoma	Established from the pleural effusion of a 69-year-old Caucasian female with metastatic mammary carcinoma (after radio- and hormone therapy) in 1970; cells were described of being positive for cytoplasmic estrogen receptors and having the capability to form domes (DSMZ 2004)	RPMI 1640 without phenolred (R7509) 10% (v/v) FBS 1% (v/v) L-glutamine 1 mM Sodium-pyruvate 0.4% (v/v) Penicillin-Streptomycine
L5178Y	Mouse T cell lymphoma	Established from an 8-month-old female DBA/2 mouse with T cell lymphoma in 1985 (DSMZ 2004)	RPMI 1640 (R0883) 10% (v/v) PS 1% (v/v) L-glutamine 1% (v/v) Sodium-pyruvate 0.4% (v/v) Penicillin-Streptomycine
LLC-PK1	Porcine proximal tubule kidney cells	Established from the kidney of a male 17lb-Hamshire-pig (Hull, Cherry et al. 1976; Perantoni and Berman 1979)	DMEM 10% (v/v) FBS 1% (v/v) L-glutamine 2.5% (v/v) Hepes 0.4% (v/v) Penicillin-Streptomycine
CaCo-2	Human colonic carcinoma cell line	Established from the primary colon tumour (adenocarcinoma) of a 72-year-old Caucasian male in 1974 (Rousset 1986)	DMEM 10% (v/v) FBS 1% (v/v) L-glutamine 0.4% (v/v) Penicillin-Streptomycine

Tab. C-5 Cell lines, media and growth conditions

2.2.1 Maintenance of Cell Culture

Cells were grown in 75 cm^2 flasks (20 ml medium) or in 25 cm^2 flasks (5 ml medium). The flasks were kept in an incubator with a moistened, 5% CO_2 atmosphere at 37 °C.

The medium was changed 3 times a week, generally on Monday, Wednesday and Friday. Unless morphological or proliferation changes could be observed, cells were used for experiments until passage 20.

2.2.2 Passaging of Cells

1. Adherent Cell Lines

Adherent cell lines like MCF-7, LLC-PK1 and CaCo-2 (see table C-5) were grown until 80% confluency was reached. Then, cells were washed twice with 10 ml pre–warmed PBS. PBS was removed and 2 ml of pre-warmed 1×Trypsin-EDTA were added. After 2-10 minutes in the incubator (5% CO_2; 37℃) cells started to detach. The trypsin digestion was stopped by adding of 8 ml medium. Subsequently cells were resuspended several times.

To obtain a new sub-culture, 2 ml of the cell suspension were transferred to a new flask with 18 ml medium.

2. Non-Adherent Cells

In non-adherent cell lines like L5178Y or HL60 cells (see table C-5) the concentration of cells was determined by coulter counter. For this purpose 200 µl of the well mixed cell suspension were added to 9.8 ml isotone buffer. The number of particles between 7.5 µm and 30 µM in a volume of 0.5 ml was determined by counter.

The actual cell concentration was calculated as follows:

$$\frac{Cells}{ml} = n \cdot V_C \cdot D \cdot V_z$$

n = number of cells as determined by coulter counter
V_c= Sample volume in coulter counter
D = Dilution factor
V_z= Sample volume of the cell suspension

The volume containing the desired amount of cells (e.g. 1×10^6) was transferred to a new flask.

2.2.3 Thawing of Cells

The cryo-vial containing frozen cells was taken out of the liquid nitrogen and thawed within 2-4 minutes. The thawed cells were transferred to a 75 cm^2 cell culture flask filled with 20 ml cold medium. Cells were kept in an incubator (37℃; 5% CO_2) overnight. The next morning, cells were supplied with fresh medium.

2.2.4 Freezing of Cells

The freezing procedure was identical for all cell lines used. Freezing medium was prepared by adding 10% (v/v) DMSO to normal cell culture medium. 1×10^6 cells were centrifuged (167 × g, 5 min), the supernatant was discharged. The pellet was resuspended in 1 ml freezing medium (4°C) and transferred into a cryo-vial. The cryo-vial was placed in a freezing container filled with isopropanol and frozen at -80°C with a cooling rate of 1°C per minute. The next morning, the cryo vials were transferred into a liquid nitrogen container.

2.2.4.1 Harvesting of Cells:

1. Suspension Cells

After incubation suspension cell cultures were thoroughly mixed, the cell density determined by coulter counter and the amount desired used in the assay.

2. Adherent Cells

In the case of adherent cells, the media was removed, cells washed twice with generous amounts of PBS and detached with trypsin-EDTA (1 ml/25 cm^2 adhered cells). The trypsin digestion was stopped by adding the threefold amount of media. Afterwards cells were separated by 3 – 5 times resuspension with a multipipette. The cell density was determined by coulter counter.

2.2.5 Treatment of Cells for Testing

For toxicity testing of uremic toxins cells were seeded with a density of 2×10^5 cells/ml media (suspension cells; HL60, L5178Y) or 4×10^4 cells/cm^2 (adherent cells; LLC-PK1, CaCo-2). After letting the cells attach to the bottom of the flask or simply adjust to the new media for 2 – 3 h the test substance was added. The concentration of stock solutions for each test was chosen to result in a final solvent concentration of 0.1% (ethanol), 1% (DMSO) or 2% (ddH_2O) in the media. These solvent concentrations were known to cause no effect in the cell culture.

For the toxicity testing of eluates L5178Y cells were seeded with a density of 2×10^5 cells/ml media. After they had adjusted to the new media, eluates were added to a final concentration of 2% (ethanol containing eluates) or 4% (ddH_2O eluates).

3 Toxicological Test

3.1 Frequent Test Substances

Frequent test substances are listed in Tab. C-6.

Test substance	Manufacturer	Solvent	Stock solution
5-Aza-2'-deoxycytidine	Sigma-Aldrich, Taufkirchen, Germany, Cat. No: A3656	DMSO	250 µM
5-Aza-cytidine	Sigma-Aldrich, Taufkirchen, Germany, Cat. No: A2385	DMSO	250 µM
Bisphenol A (BPA)	Sigma-Aldrich, Taufkirchen, Germany, Cat. No: 239658	Ethanol	1 mM
DL-Homocysteine (Hcy)	Sigma-Aldrich, Taufkirchen, Germany, Cat. No: 53510	ddH_2O	500 mM
H_2O_2	Sigma-Aldrich, Taufkirchen, Germany, Cat. No: 31642	ddH_2O	50 mM
Homocysteine-Thiolactone (Hcy-T)	Sigma-Aldrich, Taufkirchen, Germany, Cat. No: H6503	ddH_2O	500 mM
Leptin	Sigma-Aldrich, Taufkirchen, Germany, Cat. No: L4146	DMSO	1 mg/ml
Methyl-Methane-sulfonate (MMS)	Sigma-Aldrich, Taufkirchen, Germany, Cat. No: M4016	DMSO	50 µg/ml
Mitomycin C (MMC)	Sigma-Aldrich, Taufkirchen, German, Cat. No: M4287	ddH_2O	12.5 µg/ml
$NaAsO_2$	Sigma-Aldrich, Taufkirchen, Germany, Cat. No: S7400	ddH_2O	10 mM

Tab. C-6 Frequent test substances

Additional toxicity tests were performed with the serum of HD patients with elevated MN frequency. The serum was obtained through another study (Treutlein, to be submitted).

3.2 Cytotoxicity

3.2.1 Proliferation

3.2.1.1 Theoretical Background

One parameter of cytotoxicity which can be obtained fast and easily is cell proliferation. A reduction of proliferation is an indicator for either direct cytotoxic effect by induction of apoptosis or necrosis or indirectly by a diminished proliferation rate.

3.2.1.2 Procedure

After seeding of cells and addition of test substances, cells were incubated for 24 h to 48 h. Thereafter cells were harvested and the cell number determined by coulter counter.

3.2.2 BrdU Incorporation Assay

3.2.2.1 Theoretical Background

The BrdU incorporation assay can be used to evaluate changes in the cell cycle - e.g. cell cycle arrest - which can be caused by toxic substances. In this assay, BrdU - an analogue of the DNA precursor thymidine - is incorporated into newly synthesized DNA of cells entering and progressing through the S-phase (DNA synthesis) (Becton 2005). The incorporated BrdU can then be stained by an anti-BrdU antibody. The combination with a total DNA staining by 7-amino-actinomycin (7-AAD) permits the characterization of the cells in regard to their cell cycle position (G0/G1, S or G2/M phase).

3.2.2.2 Materials and Buffers

- *BrdU Flow Kit* (BD Pharmingen, Heidelberg, Germany, Cat. No. 559619)
- *Staining buffer:* 3% FBS, 0.9% sodium acid in PBS

3.2.2.3 Procedure

1. Incubation of Cells with Test Substance:

Cells were seeded with a density of 2×10^5 cells/ml and the test substance added. To ensure that the cells were in the exponential growth phase during the assay, cell density was readjusted to 2×10^5 cells/ml, 10 h prior to the actual start of the assay. The new test substance was added and the cells kept in the incubator (5% CO_2; 37 °C) for the remaining incubation period.

2. Labelling of Cells with BrdU:

10 h later BrdU was added to the media to give a final concentration of 10 µM BrdU. During BrdU incorporation cells were kept in an incubator (5% CO_2; 37 °C). After 30 min incorporation time, cells were centrifuged (200×g) and washed with 1 ml

staining buffer per sample. During incorporation time kit components were diluted according to the manual.

3. Fixation and Permeablisation of Cells:

After an additional centrifugation step, the supernatant was discharged and the cells resuspended in 100 µl BD Cytofix/Cytoperm™/sample. The cells were incubated on ice for 30 min. Next, cells were washed with 1 ml BD Perm/Wash buffer™. This was followed by an additional permeabilisation step by incubation with 100 µl BD Cytoperm Plus buffer™ for 10 minutes on ice. After an additional washing step cells were re-fixated for 5 minutes with 100 µl BD Cytofix/Cytoperm™/sample; followed by another washing step.

4. Treatment of Cells with DNase:

Cells were treated with DNase (100 µl DNase/sample) to expose incorporated BrdU. Next, cells were incubated in a 37°C water bath for 1 h.

5. Staining of BrdU with Fluorescent Antibodies:

After an additional washing step the BrdU was stained with 50 µl FITC labelled Anit-BrdU-antibody (1:50 in PBS) for 20 min at room temperature. Surplus antibody was removed by washing the cells with BD Perm/Wash buffer™.

6. Staining of total DNA:

The total DNA was stained by resuspending the cells in 20 µl 7-AAD solution included in the kit. For analysis 1 ml staining buffer was added.

7. Flow Cytometric Analysis:

The flow cytometric analysis was performed at a run of less than 400 events/sec. 15,000 cells were analysed per sample. The cells were depicted in a dot blot forward scatter (FSC, x-axis) versus side scatter (SSC, y-axis). The FSC is a parameter for the size of the cells, the SSC for the inner granularity of cells. The cell population was focused by adjustment of the forward scatter (FSC).

In a second dot plot the 7-AAD fluorescence (x-axis) was plotted versus FITC anti-BrdU fluorescence (y-axis). 7-AAD fluorescence - representing the staining of the whole DNA - was measured on channel FL3 (red fluorescence, 670 nm bandpass filter). FITC anti-BrdU fluorescence – representing the staining of integrated BrdU into newly synthesized DNA – was measured on channel FL1 (green fluorescence,

530 nm bandpass filter). In order to quantify the cell cycle positions region gates were applied. This allowed the discrimination between subsets of cells that were apoptotic (sub G1/G0) or resident in G0/G1, S or G2 + M phases of the cell cycle and had recently synthesized DNA (Fig. C-1). The optimum settings were determined with the untreated control cells.

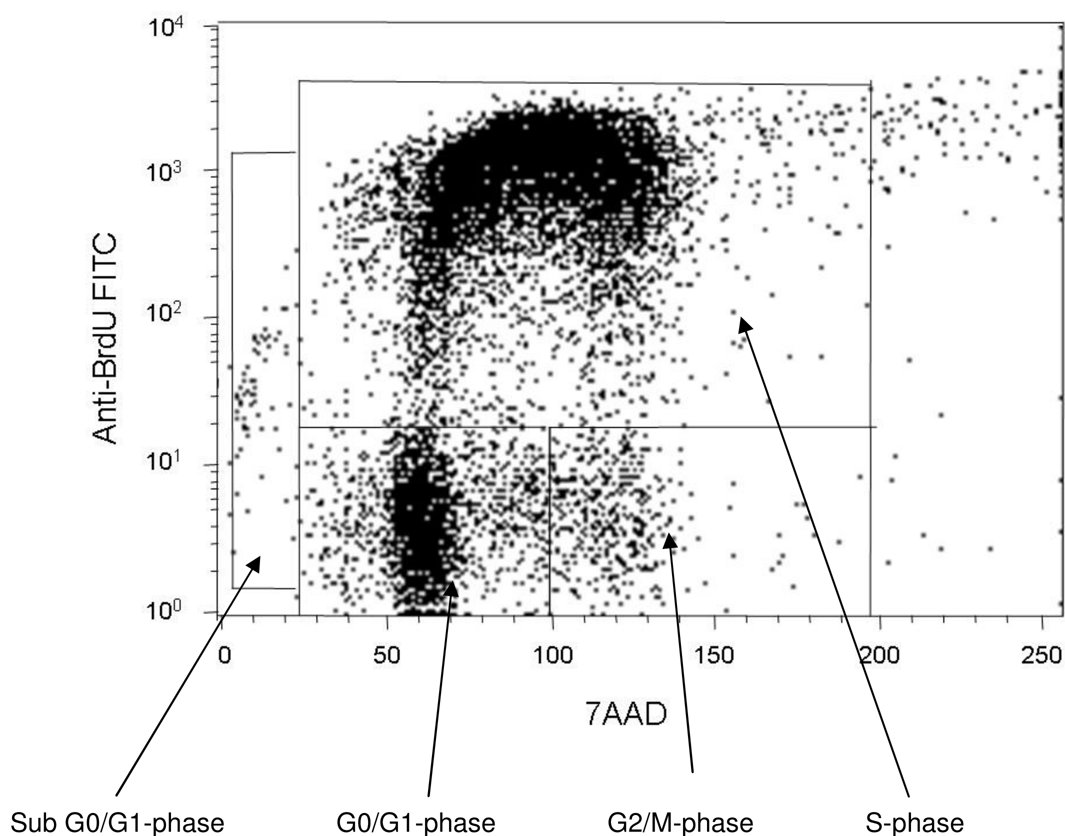


Fig. C-1 Exemplary dot plot of a quantitative cell cycle analysis of control cells, stained for incorporated BrdU and total DNA levels

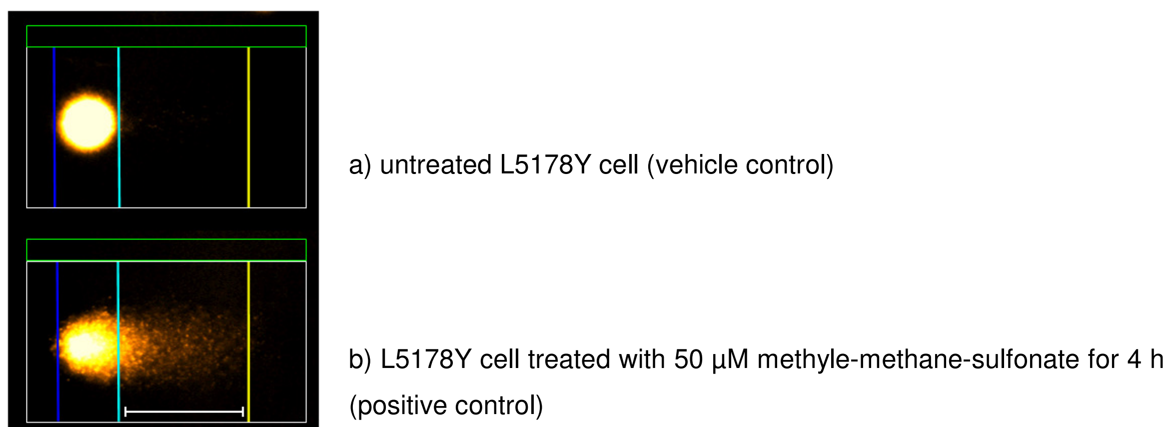
3.3 Genotoxicity

3.3.1 Comet Assay (Single-Cell Gel Test)

3.3.1.1 Theoretical Background

The comet-assay or single-cell gel test is a simple, sensitive and fast test for studying DNA damage and DNA repair (Speit and Hartmann 1998). In this method a small amount of cells are embedded into a thin layer of agarose on a microscope

slide. Subsequently the cells are lysed, electrophoresed and stained by a DNA-intercalating dye. Cells with more DNA damage display a higher migration of chromosomal DNA away from the nucleus (“head”) towards the anode, thereby forming a “tail”. This results in the typical form of a comet (Fig. C-2).



a) untreated L5178Y cell (vehicle control)

b) L5178Y cell treated with 50 µM methyle-methane-sulfonate for 4 h (positive control)

Fig. C-2 Exemplary pictures of cells in the comet assay

The alkaline version of this test – which was used in this PhD-thesis – was first described by Singh and co-workers in 1988 (Singh, McCoy et al. 1988). This test is used to monitor single-strand breaks and alkali-labile sites of the DNA. Several other variations have been described which allow e.g. the detection of cross links (Pfuhler and Wolf 1996).

The advantages of the comet assay are the easy and fast performance, the sensitivity, the need for extremely small samples and applicability for nearly every eukaryotic cell type. Therefore this test has become widely accepted for pharmaceutical tests.

3.3.1.2 Buffer & Solutions:

- *1.5% Agarose* (Agarose MEEO, Carl Roth GMBH, Karlsruhe, Germany, Cat. No: 2268.2) in PBS
- *0.5% low melting point (LMP) agarose* (Agarose Typ VII, Sigma-Aldrich, Taufkirchen, Germany, Cat. No: A4018) in PBS
- *Lysis buffer:* 2.5 M NaCl, 100 mM EDTA, 10 mM TRIS, 1% w/v N-Lauroyl-sacrosine sodium salt
- *Lysis solution*: 1% Triton, 10% DMSO in lysis buffer
- *Electrophoresis buffer*: 0.3 M NaOH, 1 mM EDTA

- *Tris buffer:* 0.4 M TRIS, pH 7.5
- *Propidium iodide solution*: 20 µg PI in double-distilled water (ddH_2O)

3.3.1.3 Procedure:

1. Preparation of Slides and Buffers:

Superfrost slides were pre-coated with a layer of 1.5% agarose. Lysis solution and electrophoresis buffer were prepared and stored at 4°C for at least 1 h prior to use. The 0.5% LMP agarose was heated in the microwave oven and place in a 37°C water bath.

2. Harvesting of Cells:

After incubation the period cells (HL60 or L5178Y) were harvested and the cell density was adjusted to 0.8 - 1.2 × 10^6 cells/ml

3. Coating of Slides with Cells:

For each sample, two slides were prepared. Thereby 180 µl of the LMP-agarosis solution were mixed with 20 µl cell suspension in a pre-warmed Eppendorf tube. 45 µl of this mixture were dropped on the pre-coated agarose slide and covered with a cover slip. After the agarose had hardened the cover slip was removed.

4. Lysis:

The slides were transferred to a cuvette filled with lysis solution (4°C). Lysis was performed at 4°C in the dark for 1 - 18 h.

5. Unwinding of DNA and Electrophoresis:

After lysis, slides were placed in a horizontal electrophoresis unit, filled with electrophoresis buffer. The slides were left in the shaded unit for 20 min in order to allow for unwinding of the DNA. Then electrophoresis at 25 V and 300 mA for 20 min followed.

6. Neutralization and Staining:

After electrophoresis slides were neutralized in 0.4 M tris buffer for 5 - 10 min. Slides were stained with 15 µl PI solution and covered with a cover slip. Slides were stored at 4°C under humid conditions until analysis.

7. Analysis:

A fluorescence microscope (Nikon, Labphot 2 A/L, Nikon, Germany) equipped with a camera (Cohn-High Performance, CDD Camera, Intas Sciences, Germany) and Komet 5 software (Kinetic Imaging, UK) was used to analyse the slides. The percentage of DNA in the tail of the comet was used to determine DNA damage. Two slides per sample were evaluated by analysing 25 cells per slide.

3.3.2 Micronucleus Test

3.3.2.1 Theoretical Background

Micronuclei (MN) are DNA-containing structures which are formed during mitosis and result from chromosomal breaks lacking centromers and/or whole chromosomes incorrectly distributed during mitosis. At telophase a nuclear envelope forms around these chromosomes or fragments, thereby forming nucleus-like structures, except that they are smaller (see Fig. C-3).

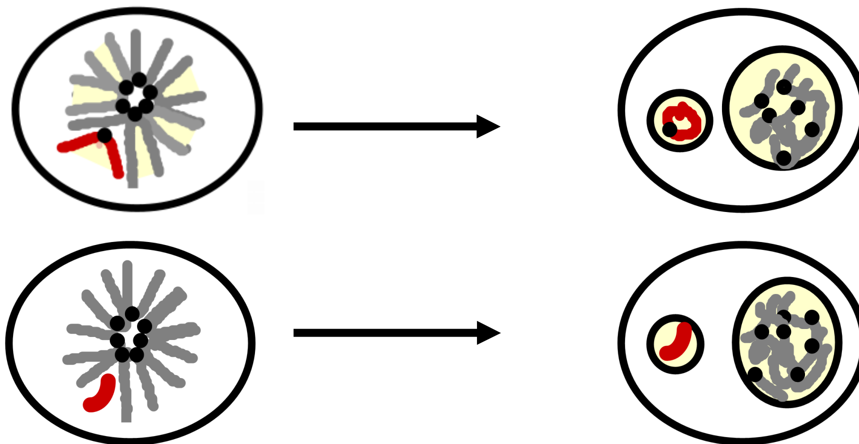


Fig. C-3 Schematic formation of micronuclei
During mitosis chromosomal breaks or chromosomes lacking centromers are incorrectly distributed. While the cell undergoes telophase, a nuclear envelope forms around these chromosomes or fragments thereby forming nucleus like structures - micronuclei

The MN represent a subgroup of all chromosomal aberrations, which makes the MN frequency test a widely accepted method for *in vitro* and *in vivo* genotoxicity investigation and human biomonitoring studies (Miller, Potter-Locher et al. 1998; Fenech 2000; Kirkland, Henderson et al. 2005). *In vitro* MN can be induced by a variety of genotoxic effects, for instance double strand breaks, oxidative stress, inhibition of the spindle formation or even interference with the DNA methylation.

It is evident that MN can only be formed by dividing cells; therefore the assay can not be used in non-dividing cell populations. Consequently the MN test was advanced and the cytokinesis-block micronucleus assay developed (Fenech and Morley 1985; Fenech and Morley 1985). In this assay cells undergoing nuclear division are blocked from cytokinesis using the inhibitor of actin polymerisation cytochalasin-B (Cyt B). Consequently the cells which have undergone one mitosis can be distinguished from the rest by their binucleated appearance. Usually the number of MN per 1000 binucleated cells is the determined.

The cytokinesis-block micronucleus assay can be also used to measure additional reactions towards cytotoxic and genotoxic events, like: necrosis, apoptosis, nucleoplasmatic bridges and cytostasis (Fig. C-4)

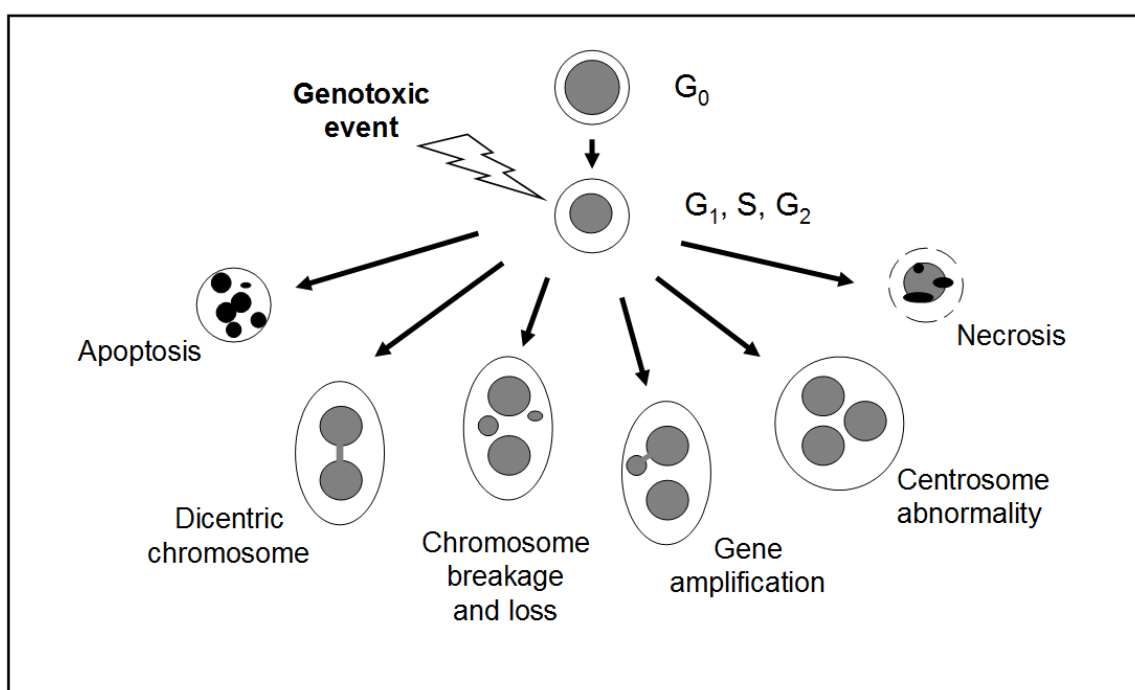


Fig. C-4 Fate of cells following exposure to cytotoxic/genotoxic agents (modified after Fenech 2002).

3.3.2.2 Materials:

- *Cyt B stock solution*: 1 mg/ml cytochalasin B (Sigma-Aldrich, Taufkirchen, Germany, Cat. No: C6762) in DMSO.
- *Staining solutions:*

  Acridine orange stock solution: 0.1% w/v acridine orange in ddH_2O

  Working solution: 6.3% v/v acridine orange stock solution in Soerensen buffer
- *Soerensen buffer:* 15 mM $Na_2HPO_4 \times 2\ H_2O$; 15 mM $KH_2PO_4 \times H_2O$; pH 6.8

3.3.2.3 Procedure:

1. Treatment of Cells with Cytochalasin B (optional):

Cytochalasin B (Cyt B) treatment was conducted in case the test substance had an effect on cell proliferation. After treatment of cells with the test substance 4 µg/ml Cyt B were added to the media. After a 24 h incubation period, cells could be harvested for cytospin preparation.

2. Preparation of Slides:

After the incubation period cells were harvested. Approximately 50,000 cells were placed on microscopic slides by cytocentrifugation (5 min, 200 × g). In order to ensure sufficient slides for analysis, 4 slides per treatment sample were produced. After a brief quality control by light microscopy (magnification: 100 ×), slides were fixed with ice-cold methanol (-20 °C) for at least 2 h.

3. Staining:

Immediately prior to analysis cells were removed from the methanol containing vial and transferred to a cuvette filled with acridine orange working solution. After 3 - 5 minutes staining, the residue acridine orange was removed by two subsequent washing steps in Soerensen buffer for 5 min. Thereafter slides were covered with a cover slip and placed in a dark, humid chamber.

4. Analysis of Cells (without Cyt B:)

Slides were analysed using a fluorescence microscope with an excitation wavelength of 450 - 490 nm (magnification: 500 ×).

Two slides per sample were analysed by counting 1000 cells per slide. The value obtained was MN/1000 cells. Depending on the problem additional parameters like number of apoptotic cells or number of mitoses were scored simultaneously.

5. Analysis of Cells treated with Cyt B:

Slides were analysed using a fluorescence microscope with an excitation wavelength of 450 - 490 nm (magnification: 500 ×). Two slides per sample were analysed by counting 1000 cells per slide. The following parameters were determined:

- number of mononucleated cells
- number of binucleated cells
- number of polynucleated cells
- number of apoptotic cells
- number of cells undergoing mitosis

In order to determine the micronucleus frequency, 1000 binucleated cells were counted and the number of MN per binucleated cells determined.

3.3.3 Determination of DNA-Cytosine Methylation by Flow Cytometry

3.3.3.1 Theoretical Background:

DNA methylation is a chemical modification of the DNA by covalent addition of a methyl group to the 5'carbon of cytosine within the context of the CpG nucleotide (s. introduction). Changes in the overall DNA methylation can be analysed by a flow cytometric analysis of DNA stained with an anti-5-methyl-cytosine antibody.

3.3.3.2 Materials

- *Fixation buffer*: 0.25% Paraformaldehyde (Sigma-Aldrich, Taufkirchen, Germany, Cat. No: 158127) in PBS
- *Wash buffer:* 1% BSA, 0.2% Tween 20 in PBS
- *88% Methanol* in PBS
- *2 N HCl* (Carl Roth GmbH, Karlsruhe, Germany)
- *Staining solution:* 0.1% BSA in PBS

- *Neutralizing buffer:* 0.1 M Borate solution (Sigma-Aldrich, Taufkirchen, Germany, Cat. No: A7906); pH adjusted to 8.5 with NaOH
- *Stop solution:* Wash buffer with 10% FBS
- *Primary antibody:* anti-5-methyl-cytosine (Calbiochem, San Diego, USA, Cat. No: 16233 D3), stock solution: 1 mg/ml; final concentration: 2 µg/ml.
- *Secondary antibody:* FITC conjugated goat anti mouse antibody (Dianova, GmbH, Hamburg, Germany, Cat. No: 115-095-003); stock solution: 1.5 mg/ml; working concentration: 1:50 in wash buffer.
- *Propidium iodide 1 mg/ml*

3.3.3.3 Procedure:

1. Treatment of L5178Y Cells:

Given that detectable changes in DNA methylation can require more than one cell cycle, cells were treated for 72 h or a minimum of 3 cell cycles. Every 24 h media was changed, cell density adjusted to 2×10^5 cells/ml and new test substance added. Cells incubated with the known methylation inhibitor 5-aza-cytidine served as positive control.

2. Harvesting of Cells:

After the incubation period 1×10^6 cells were transferred into falcon tubes. Cells were centrifuged (250 × g, 5 min) and washed twice with PBS.

3. Fixation:

The pellet was resuspended with 500 µl fixation buffer and vortexed briefly. Cells were incubated at 37°C for 10 minutes and additionally on ice for 10 minutes. During gentle vortexing 4.5 ml ice cold methanol were added und the suspension was left at -20°C for 15 minutes. Cells were centrifuged (250 × g; 5 min) washed twice with 2 ml washing buffer per sample.

4. DNA Denaturation and Neutralization:

Cells were resuspended in 1.5 ml HCl and incubated in a water bath (37°C) for 25 min. To neutralize HCl 1.5 ml borate buffer were added. The mixture was kept at room temperature for 10 min. Thereafter, cells were washed twice with 2 ml washing buffer.

5. Staining:

Prior to staining cells were immersed in 2 ml stop solution (30 min; 37°C). Thereafter, cells were centrifuged (250 g; 5 min) and washed with 2 ml washing buffer. Cells were mixed gently with 300 µl primary antibody solution. The suspension was incubated in a water bath (37°C) for 40 minutes. Subsequently, two washing steps with 2.5 ml washing buffer followed. The cells were resuspended with 300 µl secondary antibody solution and incubated in a water bath (37°C) for 40 min. After two additional washing steps with 2.5 ml washing buffer, cells were resuspended with staining solution.

6. Flow Cytometric Analysis:

Flow cytometric analyses were performed at a run of approximately 400 events/sec. 20,000 cells were analysed each sample. The cells were depicted in a dot blot FSC (x-axis, cell size) versus SSC (y-axis, inner granularity). The cell population was focused by adjustment of the FSC. The optimum settings were determined with untreated control cells.

In a histogram the FITC fluorescence (x-axis) was plotted versus the number of cells (y-axis). FITC anti-5'methyl-cytosine – representing the staining of DNA methylation – was measured on channel FL1 (green fluorescence, 530 nm band pass filter) (Fig. C-5). A shift of the peak to the left shows a decrease of bound anti-5-methyl-cytosine antibody. This indicates a decrease of overall DNA methylation.

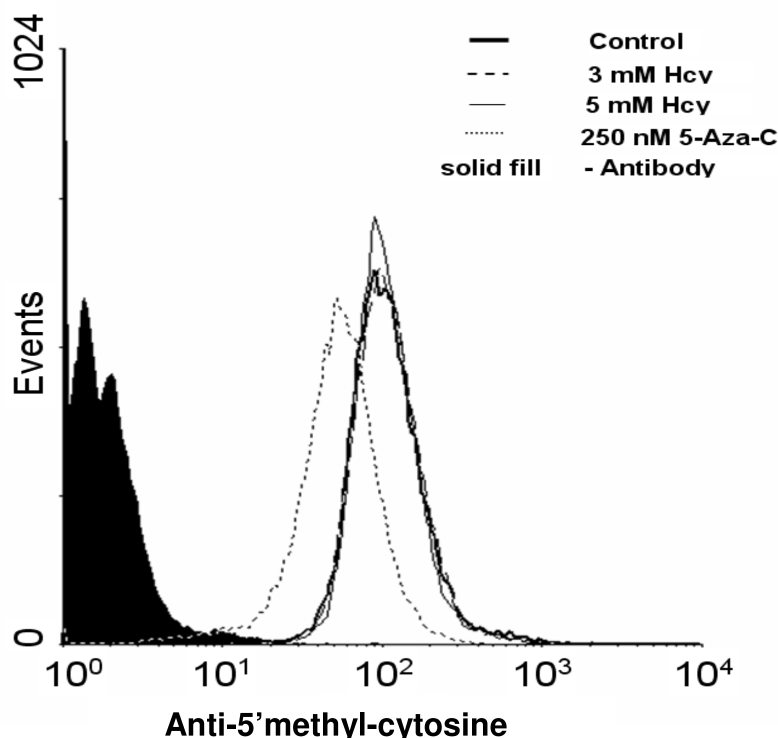


Fig. C-5 Exemplary histogram of a flow cytometric analysis of L5178Y cells, stained against 5'-methyl-cytosine. The solid filled graph matches cells not stained by antibody; the non-filled graphs match cells stained by antibody.

3.3.4 Determination of DNA-Cytosine Methylation by LC-MS/MS

3.3.4.1 Theoretical Background:

Another possibility to determine overall DNA methylation is by HPLC-MS/MS. In this method the DNA of treated cells is isolated and digested to the 2'deoxyribonucleosides. The amount of 5-methyl-2'deoxycytidine (5-mdCyd) and 2'deoxyguanosine (dGuo) is analyzed by LC-MS/MS. The DNA cytosine methylation is determined as the quotient 5-mdCyd/dGuo based on the assumption that the sum of deoxycytidine and 5-mdCyd equals dGuo in genomic DNA. The DNA-Cytosine methylation measurement by LC-MS/MS was performed by Andreas Brink (method described in (Fink, Brink et al. 2007).

3.3.4.2 Materials:

- *DNA isolation kit* (Nucleobond® AX, Macherey-Nagel, Dueren, Germany)

- *C18 HPLC column* (Reprosil Pur ODS, 2.0 mm x 150 mm; 5 µm; Dr. Maisch, Ammerbuch, Germany)
- *Analyst 1.4.1* (API 3000, Applied Biosystems)
- *5-methyl-2'deoxycytidine* standard solution (Sigma-Aldrich GmbH, Munich, Germany)
- *2'deoxyguanosine* standard solution (Sigma-Aldrich GmbH, Munich, Germany)
- *Buffer A:* 25 mM $CH_3CO_2NH_4$ (Sigma-Aldrich GmbH, Munich, Germany, Cat. No: A1542), 1 mM $ZnCl_2$ in ddH_2O; pH 5.1
- *Buffer B:* 100 mM NH_4HCO_3 (Sigma-Aldrich GmbH, Munich, Germany, Cat. No: A6141), pH 8.0
- *Buffer C:* 1 M $CH_3CO_2NH_4$ (Sigma-Aldrich GmbH, Munich, Germany, Cat. No: A1542), 45 mM $ZnCl_2$ in ddH_2O; pH 5.1
- *Buffer D:* 1.5 M NH_4HCO_3 (Sigma-Aldrich GmbH, Munich, Germany, Cat. No: A6141), pH 8.0
- *Nuclease P1:* (Sigma-Aldrich GmbH, Munich, Germany, Cat. No. N8630), working nuclease: dissolved in buffer A to a final concentration of 1 unit/µl.
- *Alkaline phosphatase:* (Calbiochem, San Diego, California, USA, Cat. No. 524576); working solution: dissolved in buffer B to a final concentration of 200 units/ml
- *Amicon Ultafree®-MC centrifugal units:* (Millipore, Schwalbach, Germany, Cat. No. UFC3LCC00)

3.3.4.3 Procedure:

1. Treatment of Cells:

Given that detectable changes of DNA methylation can require more than one cell cycle, cells were treated for 72 h or at least 3 cells cycles. Every 24 h the media was changed, cell density adjusted to 2×10^5 cells/ml and the new test substance added.

2. DNA Isolation:

After incubation the DNA was isolated according to the DNA isolation kits manual. In brief, 5×10^6 cells were washed twice with PBS and treated with the buffer supplied to disrupt the cell membrane. Proteins were digested by addition of proteinase K (20 mg/ml) followed by an incubation step (50°C, 60 min). After

equilibration the DNA was loaded onto a cartridge, washed three times and eluted again. The DNA was precipitated over night at 4°C by the addition of isopropanol. The resulting pellet was dissolved in 10 µl ddH_2O.

3. Photometrical Analysis of DNA Quantity:

1 µl of dissolved DNA was diluted with 99 µl ddH_2O and transferred to an acryl cuvette. DNA content was determined by measuring the absorption at 260 nm. Triplicates for each sample were prepared and measured twice. The DNA content was calculated applying the following equation:

$$(Absorption_{260} \times \text{dilution factor} \times 50)/1000 = \text{DNA } \mu g/\mu l$$

Approximately 5×10^6 cells resulted in 5 to 20 µg of DNA.

The DNA concentration of the samples was adjusted to approximately 1 µg DNA/µl.

4. DNA Hydrolysis:

DNA was digested to the 2'-deoxyribonucleosides by adding 1 µl of buffer C to 20 µl sample (≈ 20 µg DNA). In order to liberate the nucleotides from the DNA the sample was incubated with 2 µl Nuclease P1 (0.1 units per µg DNA) for 120 min at 40°C. Then, 2 µl buffer D as well as 2 µl alkaline phosphatase (0.02 units/µg DNA) were added to the sample to catalyze the hydrolysis of 5′-terminal phosphates of DNA. The incubation was performed at 40°C for 60 min. Thereafter, the sample was centrifuged (20 min; 7200 × rpm, 4°C) in a 5000 atomic mass units (amu) cut-off filter tube to remove the enzymes.

5. LC-MS/MS Analysis:

Prior to analysis of 5-mdCyd and dGuo the samples were diluted with ddH_2O (1:100). 10 µl were injected on a C18 HPLC column Reprosil Pur ODS using an Agilent 1100 autosampler and an Agilent 1100 HPLC-pump. The samples were separated by gradient elution with water containing 0.1% formic acid (Solvent A) and acetonitril (Solvent B) employing the following conditions: 10% B linear to 40% in 3 min, then linear to 100% within 2.5 min at a flow rate of 300 µl/min. The eluent was analysed in the multiple reactions monitoring modus using a triple-stage quadruple mass spectrometer equipped with an electrospray ionization source, controlled by

Analyst 1.4.1. The ion spray voltage was 3400 V, the source temperature 400℃. The transitions monitored with a dwell time of 0.1 seconds each for 5-mdCyd and dGuo, were m/z 242 → 126 and m/z 268 → 152. Declustering potentials were set to 16 and 20 V, focusing potentials to 130 and 200 V for 5-mdCyd and dGuo respectively. The collision energies were set to 55 and 30 V for 5-mdCyd and dGuo, the collision cell exit potentials were 10 and 15 V respectively.

Calibration curves were constructed using 5-mdCyd and dGuo standard solutions. DNA cytosine methylation was determined as 5-mdCyd/dGuo using dGuo as internal standard based on the assumption that the sum of dCyd and 5-mdCyd equals dGuo in genomic DNA.

3.4 Oxidative Stress Measurement

3.4.1 Reactive Oxygen Species Measurement:

3.4.1.1 Theoretical Background:

2`,7`-Dichlorofluorescein diacetate (DCFH-DA) can be used to measure intracellular oxidant production (Robinson, Bruner et al. 1988). DCFH-DA is a non-fluorescent, membrane permeable substance which is enzymatically hydrolysed to the non-fluorescent DCFH by intracellular esterases. In the presence of ROS, DCFH is easily oxidized to the highly fluorescent 2`,7`-dichlorofluorescein (DCF). The DCF signal is analysed by flow cytometry, giving information about the amount of oxidative stress in the cells.

3.4.1.2 Materials:

- *2`,7`-Dichlorofluorescein diacetate* (Serva, Heidelberg, Germany, Cat. No:19353) working solution: 20 mM DCFH-DA in DMSO
- *1% PBS-BSA:* 1% BSA in PBS
- *Propidium iodide solution*: PI working solution: 100 µg/ml in ddH_2O

3.4.1.3 Procedure:

1. Treatment with DCFH-DA:

15 min prior to incubation with the test substance, DCFH-DA was added to the media at a final concentration of 10 µM.

2. Preparation of Cells for Analysis:

Cells were treated with the test substance and harvested after the desired incubation period. Cells were washed twice with BSA-PBS and finally cells resuspended in 1 ml BSA-PBS.

3. Staining with Propidium Idiodide:

10 µl PI working solution were added to the cells, resulting in a final concentration of 1 µg/ml.

4. Flow Cytometric Analysis:

The flow cytometric analyses were performed at a run of 400 - 800 events/sec. The fluorescence of 20,000 cells was measured. The cells were depicted in a dot blot FSC (x-axis, cells size) versus SSC (y-axis, cell granularity). The cell population was focused by adjustment of the FSC.

In a second dot plot the PI fluorescence (x-axis) was plotted versus the number of cells. PI fluorescence was measured on channel FL3 (red fluorescence, bandpass filter 670 nm). PI is not able to diffuse through membranes of living cells; therefore living cells can be distinguished from dead cells through the lack of red fluorescence.

A gate was put around the living cells in the plot and a histogram showing their DCF fluorescence (x-axis) *vs*. number of cells was constructed. DCF fluorescence represents the amount of ROS within the cell. It was measured on channel FL1 (green fluorescence, bandpass filter 530 nm). A shift of the generated peak to the right indicated an increase of ROS within the cells (Fig. C-6).

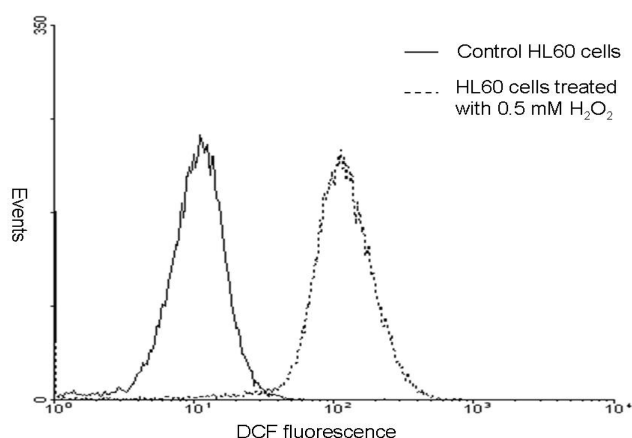


Fig. C-6 Exemplary oxidative stress measurement of HL60 cells using DCFH-DA as dye Increased oxidative stress causes increased DCF fluorescence and therefore a shift the peak to the left.

3.5 GSH/GSSG – Assay

3.5.1.1 Theoretical Background:

Reduced glutathione (GSH) is one of the major antioxidants in nucleated cells. It provides the reducing equivalent for the reduction of hydrogen peroxide and lipid hydroperoxides. During this process GSH is oxidised to GSSG. GSSG is then recycled to GSH by the glutathione reductase and NADPH.

This principle is used for the quantitative determination of total GSH. The method was first described by Tietze (Tietze 1969). It employs 5`5-dithiobis-2-nitrobenzoic acid (DTNB), which reacts with GSH resulting in a change of colour (Fig. C-7). This change is proportional to the GSH and GSSG concentrations and can therefore be used to quantify the total GSH concentration.

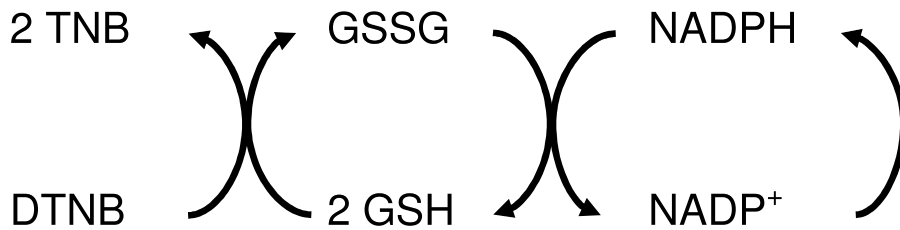


Fig. C-7 Reactions of the GSH-GSSG assay

GSH = reduced glutathione; GSSH = oxidized glutathione; DTNB = 5`5-dithiobis-2-nitrobenzoic acid; TNB = 2-Nitro-5-thiobenzoic acid; NADPH = Nicotinamide adenine dinucleotide phosphate (reduced); $NADP^+$ = Nicotinamide adenine dinucleotide phosphate (oxidized)

3.5.1.2 Materials:

- *0.1 mM Na_2HPO_4 buffer* in ddH_2O, pH 7.5
- *60 mM NADPH:* (Sigma-Aldrich GmbH, Munich, Germany, Cat. No: N-1630*)* in 0.01 M NaOH
- *50 mM EDTA* (Carl Roth GmbH, Karlsruhe, Germany, Cat. No: 8040.3) in ddH_2O
- *25 mM DTNB* (Sigma-Aldrich GmbH, Munich, Germany, Cat. No: D-8130) in ethanol
- *1% Sulfosalicylacid [m/m]*
- *Glutathione reductase* (Roche, Mannheim, Germany, Cat. No: 10.105.768. 001)
- *10 mM reduced glutathione* (Sigma-Aldrich GmbH, Munich, Germany, Cat. No: G-4251)
- *Reductase solution (for 10 measurements):*

  1.5 ml 100 mM phosphate buffer
  30 µl 50 mM EDTA
  15 µl 60 mM NADPH
  8 µl reductase
  1447 µl water

3.5.1.3 Procedure:

1. Sample Preparation:

   1.5×10^6 cells were centrifuged (200 × g, 5 min, 4°C) and washed twice with cold PBS. The pellet was resuspended in 400 µl sulfosalicylacid in order to disrupt the cell membrane. Cells were left on ice for 15 minutes and centrifuged (5000 × g, 3 min, 4°C). The pellet was discargded; the cell extract used for measurement.

2. Photometric Measurement:

   Prior to sample measurement a GSH calibration curve was established using 8 calibration points (250 µM, 500 µM, 750 µM, 1000 µM, 1250 µM, 1500 µM, 1750 µM, 2000 µM). For the sample measurement 20 µl cell extract were added to 260 µl phosphate buffer. Then 20 µl DTNB and 300 µl reductase solution were added. The kinetic was measured at 410 nm for 90 sec.

3. Data Analysis:

   The slope of the kinetic was calculated using Microsoft Excel. The actual GSH concentration was determined by comparison to the calibration curve.

3.6 Apoptosis

Apoptosis - the process of cell suicide – is an active process which can be triggered by a multitude of external and internal signals, e.g. toxic cell injury. In contrast to necrosis, apoptosis follows orderly steps. After the stimulus a signalling cascade is set off. One of the first consequences is the destruction of the cytoskeleton, which leads to shrinking and rounding of the cells. Furthermore the chromatin undergoes condensation into compact patches against the nuclear envelope, followed by DNA fragmentation. Moreover, the cell membrane forms irregular buds and releases apoptotic bodies.

3.6.1 Bisbenzimide Staining:

3.6.1.1 Theoretical Background:

One important feature of cells undergoing the apoptotic process is the irreversible condensation of chromatin, followed by the fragmentation of the nucleus. These

changes can be analysed microscopically after staining of the total DNA with DNA-binding dyes like bisbenzimide (Hoechst 33342), (Fig. C-8).

a) Apoptotic cell stained with Hoechst 33342 (× 1000)

b) Untreated LLC-PK1 stained with Hoechst 33342 (× 1000)

Fig. C-8 L5178Y cells stained with the Hoechst 33342 dye

3.6.1.2 Materials:

- *Bisbenzimide (Hoechst 33342):* Working solution: 50 µM in ddH_2O
- *Mounting media:* Vectashield® Mounting Medium (Linaris, Wertheim, Germany, Cat No: H-1000)

3.6.1.3 Procedure:

1. Preparation of Slides:

After harvesting, approximately 50,000 cells were placed on microscopic slides by cytocentrifugation (5 min, 200 × g). In order to ensure sufficient slides for analysis, 4 slides per treatment sample were produced. After a brief quality control by light microscopy (magnification 100 ×) slides were fixed in ice cold methanol (-20°C) for at least 2 h.

2. Staining:

Prior to analysis, cells were removed from methanol and transferred to a cuvette filled with bisbenzimide working solution. After 3 min staining, the residue bisbenzimide was removed by two subsequent washing steps with PBS for 5 minutes. A coverslip was placed on the slide with one drop of mounting media. Prepared slides were stored in a dark, humid chamber.

3. Analysis of Cells

Slides were analysed using a fluorescence microscope with an excitation wavelength of 330 - 380 nm. Two slides of each sample were analysed by counting 1000 cells per slide. The value obtained was number of apoptoses/1000 cells.

3.6.2 Annexin V Staining and FACS Analysis

3.6.2.1 Theoretical Background:

During early stages of apoptosis phosphatidylserine from the inner layer of the plasma membrane is translocated to the external surface of the cell. This phosphatidylserine can be labelled with the fluorescence-conjugated binding protein Annexin-V-Fluos. Since necrotic cells also expose phosphatidylserine due to the loss of membrane integrity, apoptotic cells have to be distinguished from necrotic ones by the simultaneous staining with PI. PI is a red-fluorescent molecule that stains nucleic acids. PI is membrane-impermeant, allowing discrimination between vital and vital but apoptotic cells on the on hand and necrotic cells on the other hand.

3.6.2.2 Materials:

- *Annexin-V-Fluos:* (Roche, Mannheim, Germany, Cat. No. 1828681)
- *AnnexinV/PI- staining solution*: 20 µl Annexin-V-Fluos, 20 µl PI solution, 960 µl 1x binding buffer
- *Binding buffer (10×)*: 0.1 M HEPES (pH 7.4)
140 mM NaCl
25 mM $CaCl_2$
- *Propidum iodide*: 1mg/ml

3.6.2.3 Procedure:

1. Harvesting of Cells:

Cells were resuspended and the cell number determined by coulter counter. 1×10^6 cells were transferred into a falcon tube, centrifuged (5 min, 200 × g) and washed once with 1× binding buffer.

2. Staining:

Cells were centrifuged (5 min, 200 × g) again and resuspended in 200 µl Annexin-V/ PI staining solution. After 20 minutes staining, 900 µl binding buffer were added to the cells.

3. Flow Cytometric Analysis:

The flow cytometric analysis was performed at a run of 400 - 800 events/sec. The cells were depicted in a dot blot FSC (x-axis, cell size) versus SSC (y-axis; cell granularity). The cell population was focused by adjustment of the FSC.

In a second dot plot Annexin-V-Fluos fluorescence (x-axis) was plotted versus PI fluorescence (y-axis). Annexin-V-Fluos was measured on channel FL1 (green fluorescence; 530 nm bandpass filter). PI fluorescence was measured on channel FL3 (red fluorescence, 670 nm bandpass filter). The fluorescence of 20,000 cells was acquired and the events gated into 4 quadrants (Fig. C-9).

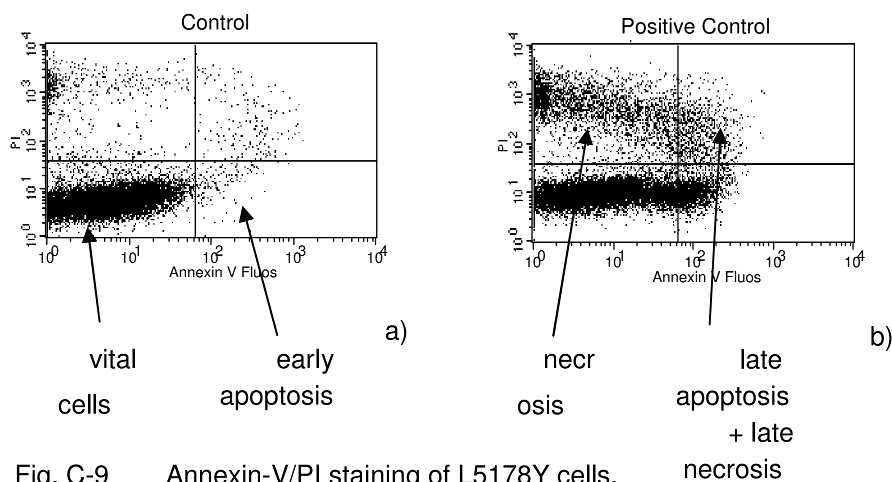


Fig. C-9 Annexin-V/PI staining of L5178Y cells.

3.7 Test for Estrogenic Activity: E-Screen

3.7.1 Theoretical Background

Several methods for *in vivo* or *in vitro* analysis of the estrogenic activity exist. One of the most popular *in vitro* tests is the so called E-screen. In this methodical approach the estrogenic activity of a substance is tested by the proliferation of the estrogen sensitive cell line MCF-7. These human breast cancer cells carry an estrogen receptor and respond to xenoestrogens by increased proliferation. In order to perform this test properly it is important to avoid any hormonal activity in the

media. Therefore the standard FBS in media is replaced by a charcoal /dextran treated FBS, which is free of hormonal activity.

3.7.2 Material

- *0.02% EDTA Solution* (Sigma-Aldrich GmbH, Taufkirchen, Germany, Cat. No: E8008)
- *Charcoal/Dextran treated FBS* (HyClone, Logan, USA, Cat. No: SH30068.02) (heat inactivated at 56°C for 30 minutes)
- *Reduced media:* Same media as for cell culture but the 10% FBS was replaced by 5% *FBS-DCC.*

3.7.3 Procedure

1. Culture of Cells for the E-Screen

MCF-7 cells were cultured like any other adherent cell line, the only difference being that the detachment of the cells occurred by 0.02% EDTA solution instead of Trypsin-EDTA. This procedure is gentler to the cells and avoids accidental destruction of the estrogen receptor on the cell surface.

2. Preparation of the E-Screens

MCF-7 cells were harvested using 0.02% EDTA solution. 12,000 cells/cm^2 were seeded in 25 cm^2 cell culture flasks. The flasks were placed in an incubator (37°C, 5% CO_2) for 24 h.

After 24 h the media was replaced by fresh reduced media and the test substances added. Three replicates of each sample were produced. After 72 h the media was changed and fresh test substance added. The cells remained in the incubator for further 72 h.

3. Harvesting of Cells:

Cells were washed twice with 5 ml with PBS and detached with 2 ml Trypsin-EDTA per flask. The trypsin stayed on the cells for up to 10 min to allow an as good detachment of the cell-cell as of the cell-matrix contacts. The trypsin was stopped by addition of 3 ml media. To ensure separation cells were resuspended with a dispenser tip.

4. Analysis:

Cells were counted by coulter counter. The values obtained were normalized to the control (100%). The percental increase of growth resembles the estrogenic activity of the substances.

3.8 Generation of Advanced Glycation End Products:

3.8.1 Theoretical Background

AGEs are a heterogenous group of compounds which are formed by non-enzymatic reactions between reducing sugars and free amino groups of proteins, followed by subsequent reactions. As it is difficult to test and characterize a heterogenous group of molecules, two model AGEs were prepared: carboxy(methyl)lysine-modified bovine serum albumin (CML-BSA) and methylglyoxal-modified BSA (MGO-BSA).

3.8.1.1 MGO-BSA Production

Material

- *0.1 M Na_2HPO_4* buffer (Merck, Darmstadt, Germany, Cat. No: 567547)
- *40% Methylglyoxal* (Sigma-Aldrich, Taufkirchen, Germany, Cat. No: M0252)
- *BSA*

Procedure

1. Production of MGO-Matrix:

2.65 g BSA were dissolved in 40 ml 0.1 M Na_2HPO_4 buffer. 10 ml of this solution were kept as control. The remaining 30 ml were mixed with 633 µl methylglyoxal. The solution and the control were filtered sterile.

2. Incubation:

The solutions were incubated for 7 days at 37°C.

3. Dialysis:

After incubation the solution as well as the control was transferred into dialysis tubing membranes. MGO-BSA was now purified by dialysis against ddH_2O for 72 h. During dialysis ddH_2O was changed several times (30 l in total).

4. Lyophilisation:

After dialysis the solutions were frozen at – 80°C and freeze-dried at - 58°C for 48 - 72 h.

5. Preparation of MGO Stock Solution:

Immediately prior to the experiment MGO was dissolved in PBS [c = 100 mg/ml].

3.8.1.2 CML-BSA Production

Materials

- *0.2 M Na_2HPO_4 buffer* (Merck, Darmstadt, Germany, Cat. No: 567547)
- *10 M NaOH*
- *1.5 M glyoxylic acid in Na_2HPO_4 buffer*
- *$NaCNBH_3$*

Procedure

1. Production of CML-Matrix:

2.65 g BSA were dissolved in 40 ml 0.1 M Na_2PO_4 buffer. 10 ml of this solution were kept as control. The remaining 30 ml were mixed with 1 ml glyoxylic acid. The solution was stirred at RT for 2 h. After adjustment of the pH to 7.4 (with 10 M NaOH), 0.238 g $NaCNBH_3$ were added. The solution and the control were filtered sterilely.

2. Incubation:

The flasks were incubated at room temperature for 2 - 3 days.

3. Dialysis:

After incubation the solution as well as the control was transferred into tubular dialysis membranes. CML-BSA was now purified by dialysis against ddH_2O for 72 h. During dialysis, ddH_2O was changed several times (30 l in total).

4. Lyophilisation:

After dialysis, the solutions were frozen at – 80℃ and freeze-dried at - 58°C for 48 - 72 h.

5. Preparation of CML Stock Solution:

Immediately prior to the experiment CML was dissolved in PBS [c = 100 mg/ml].

4 Extraction of Eluates from Various Dialysers

4.1 Conditions of Elution

The conditions of elution should resemble the real-life dialysis modalities as closely as possible. Therefore the elution was performed in an incubator at 37℃ ± 1℃, the temperature at which dialysis is performed. The eluting media of choice should feature three characteristics: they should have similar extraction properties like blood, they should not interfere with HPLC-MS/MS analysis and it should be possible to use them in toxicity tests without complicated reconditioning, which could interfere with the tests. Therefore barely volatile (e.g. oil) or saline solutions (e.g. artificial serum) could not be used. For simplified analysis ddH_2O was used. Additionally a 17.2% ethanol (EtOH) solution was used because its extraction properties towards BPA are similar to bovine serum (Haishima, Hayashi et al. 2001).

In order to prevent any contamination with ubiquitous existing substances like phthalates, only glass equipment was used. All equipment was sterilized for 8 h at 240℃.

4.1.1 Materials:

- *Eluents:*
    - HPLC grade water in glass bottles (Carl Roth GmbH, Karlsruhe, Germany)
    - 17.2% ethanol: HPLC grade ethanol in glass bottles (Carl Roth GmbH, Karlsruhe, Germany) diluted with HPLC grade water
- *Dialysers* (Tab. C-7):
    - FX60, F60S, FX80, HF80S (Fresenius Medical Care, Bad Homburg, Germany)
    - 170 H (Gambro, Hechingen, Germany)

Dialysers	Lot-Number	Potting material	Sealing ring	Housing	Membranes	Surface area
FX60	LDV15104 NBV12101	Polyurethane	Silicone	Polypropylene	Helixone® (Polysulfone – PVP blend)	1.4 m^2
F60S	LCB29101	Polyurethane	Silicone	Polycarbonate	Fresenius Polysulfone®(Polysulfone –PVP blend)	1.3 m^2
FX80	NCX01130 NGV3021	Polyurethane	Silicone	Polypropylene	Helixone (Polysulfone – PVP blend)	1.8 m^2
HF80S	NAK24160 NEK11140	Polyurethane	Silicone	Polycarbonate	Fresenius Polysulfone®(Polysulfone –PVP blend)	1.8 m^2
170 H	S-4402-H-01	Polyurethane	Silicone	Polycarbonate	Polyamide S™ (Polyamid/Polysulfone blend)	1.7 m^2

Tab. C-7: Dialysers and their properties

- *Tubing:*
    - PVC (Sis-Ter SpA, Palazzo Pignano, Italy)

4.1.2 Procedure:

1. Assembly of the Dialysis Equipment

In order to elute all leaching substances from the dialyser, the blood and the dialysate compartment were connected by 110 cm standard PVC tubes. The eluent was poured into the dialyser using only sterilized glass equipment. The approximate volume of eluent filled in the dialysers is given in Tab. C-8. Eventually, the tubes of the filled dialyser were connected to a peristaltic pump (Fig. C-10).

Fig. C-10 Assembly of the dialysis equipment

2. Dialysis:

Elution was performed in an incubator at 37 ± 1℃ with a flow rate of 230 ml eluate/min for 4 h or 24 h, respectively. The 4 h time period was equivalent to the real time of clinical dialysis, the 24 h period resembled the worst case scenario. At least 3 independent eluates with new dialysers each time were obtained at each elution condition.

3. Processing of the Eluates:

After the desired elution time the extraction was stopped. Only 51 - 64% (depending on the dialyser type) of the fluid used could be regained (Tab. C-8). This was due to absorption of eluate in the hollow fibres system.

This fluid was poured into 250 or 500 ml flasks. Thereafter the ethanol was removed by rotary evaporation at 38 - 40℃. The remaining eluate was frozen at -80℃ and lyophilised at - 58℃ for 2 - 4 days. The solid substance obtained was dissolved in 2 - 4 ml eluate, depending on the ease of solubility. Dissolving in 2 ml eluate was preferred, because this resulted in a higher concentration of leaching

substances and therefore finally in a higher concentration in the cell culture test systems. Four ml eluent were only used in case solubility in 2 ml could not be achieved.

To obtain any substance potentially left in the flask, the flask was washed with additional eluent. The analyses described below were performed with the first eluate and the second eluate.

In order to determine the quantity of contaminations originating from the treatment of the eluates and not from the dialyser, 300 ml of ddH_2O or 17.2% ethanol were filled into flasks and treated like the eluates. They served as control.

Dialysers	Eluent utilised	Eluate obtained	Percentage regained
FX60	≈ 230 ml	≈ 125 ml	≈ 54%
F60S	≈ 315 ml	≈ 200 ml	≈ 64%
FX80	≈ 285 ml	≈ 145 ml	≈ 51%
HF80S	≈ 435 ml	≈ 280 ml	≈ 64%
170 H	≈ 410 ml	≈ 250 ml	≈ 61%

Tab. C-8 Utilized and obtained eluate from different dialysers

5 HPLC-MS/MS Analysis

5.1 Full Range Scan

5.1.1 Theoretical Background

In order to get a general idea of substances detectable in the eluates, a full range scan of dialyser FX60 and F60S eluates was performed. In this method ions are not filtered so every mass can be detected.

5.1.2 Materials

- *Column:* Phenomenex Synergi 4a Hydro RP (4.0-µm, 150 x 2,0 mm, Phenomenex, USA)
- *ddH_2O:* HPLC gradient grade quality (Carl Roth GmbH& Co, Karlsruhe, Germany)
- *Acetonitrile:* HPLC gradient grade quality (Carl Roth GmbH& Co, Karlsruhe, Germany)
- *Analysis software*: Analyst 1.4.1 software (Applied Biosystems, Darmstadt, Germany)

5.1.3 Procedure

Samples (10 µl) were separated by a Phenomenex Synergi 4a Hydro RP column using Agilent 1100 autosampler and an Agilent 1100 HPLC pump. Gradient elution with water (solvent A) and acetonitrile (solvent B) was applied with the following conditions: 1 min 90% B, 10% A; for 14 minutes a linear increase up to 100% A; 5 min 100% A; for 2 min decrease of the gradient up to 10% A and 90% B. The gradient was maintained for a further 3 min. By using this gradient, hydrophilic as well as hydrophobic substances could be eluted from the column and detected later on. The flow rate was 300 µl/min. The experiments were performed on a linear ion trap mass spectrometer equipped with a TurboIon®Spray source connected to the HPLC system. To record spectral data, a vaporizer temperature of 450°C and a TurbIonSpray voltage of -4.5 kV in the in the positive ion mode were applied.

5.2 BPA Analysis

5.2.1 Theoretical Background

One of the molecules expected in the sample matrix was BPA. The analysis was performed according to Völkel et al by L-MS/MS using API 3000 (Volkel, Bittner et al. 2005).

5.2.2 Materials

- *Column:* Reprosil-Pur ODS-3 (5 µM, 150 × 4.6 mm, Maisch, Ammerbuch, Germany)
- *ddH$_2$O:* HPLC gradient grade quality (Carl Roth GmbH& Co, Karlsruhe, Germany)
- *Acetonitrile:* HPLC gradient grade quality (Carl Roth GmbH& Co, Karlsruhe, Germany)
- *Internal standard*: d_{16}-BPA (generated and provided by Dr. Völkel, Institute of Toxicology, Würzburg, Germany), (Volkel, Colnot et al. 2002)
- *Analysis software*: Analyst 1.4.1 software (Applied Biosystems, Darmstadt, Germany)

5.2.3 Procedure

Prior to analysis, eluate samples were spiked with 20 ng/ml internal standard d_{16}-BPA. Samples (10 µl) were separated by a Reprosil-Pur ODS-3 column using an Agilent 1100 autosampler and an Agilent 1100 HPLC pump. Gradient separation was performed with water (solvent A) and acetonitirile (solvent B) as solvents: 40% A / 60% B for 2 min, followed by a linear gradient to 20% A and 80% B within the next 20 min; 80% B and 20% A for a further 2 min. To record spectral data, a vaporizer temperature 450°C and a TurboIonSpray voltage of - 4.5 kV in the negative ion mode were applied. The declustering potential was set to - 40 V and N_2 was used as collision gas. For analysis of the BPA content the MS/MS transitions m/z 227/212 (BPA quantifier) and m/z 403.2 - 113.1 (d16 BPA quantifier) were evaluated. The enhanced resolution was performed at a scan rate of 250 amu/s and a fill time of 50 s. The declustering potential was set to -40 V.

Quantification of BPA was based on a calibration curve using 7 data points (0, 3.1 ng/ml, 6.2 ng/ml, 12.5 ng/ml, 25 ng/ml, 50 ng/ml, 100 ng/ml), with R^2 = 0.997. The total quantity of eluted BPA was determined by summation of BPA in eluate 1 and 2. The limit of detection (d.l.) was ca.0.57 ng/ml (signal to noise 3). The limit of quantification was ca. 3.4 ng/ml.

5.3 DEHP Analysis

5.3.1 Theoretical Background:

Several studies reported an elevation of di(2-ethylhexyl)phthalate (DEHP) levels in the serum of ESRD patients after dialysis. Therefore the eluate content of DEHP was analysed *in vitro*. DEHP analyses of eluates from extracorporal circuits were performed.

5.3.2 Materials:

- *Column:* Luna Phenyl-Hexyl column (3 µm, 150 × 4.6 mm, Phenomenex, USA)
- *Acetonitrile:* HPLC gradient grade quality (Carl Roth GmbH& Co, Karlsruhe, Germany)
- *Formic acid* (first grade, Sigma-Aldrich, Taufkirchen, Germany, Cat. No. 06440)
- *Internal standard*: Bis-(2-ethylhexyl)-phthalate PESANAL® (Sigma-Aldrich, Taufkirchen, Germany, Cat. No: 36735)

5.3.3 Procedure:

Before the analysis samples were diluted with acetonitrile containing 0.1% formic acid (1:9) additionally 100 pg/ml internal standard was added. The internal standard as well as the DEHP for the calibration curve was dissolved in acetonitrile containing 0.1% formic acid.

The diluted sample (10 µl) was separated by a Luna Phenyl-Hexyl column The analysis was carried out isocratically with 98% acetonitrile (containing 0.1% formic acid) and 2% of 0.1% formic acid. For analysis of the DEHP content the MS/MS transitions m/z 391.2/57.1 were evaluated.

The calibration curve was calculated from 5 data points (10, 50, 100, 500, 1000 ng/ml) (R<0.99). The limit of detection (d.l.) was ca 20 ng/ml (signal to noise 3). The limit of quantification was between ca 70 ng/ml (signal to noise 10).

D Results

1 Substances Extracted from Blood Circuits Containing Dialysers and Tubings

The eluates obtained from various dialysers were concentrated by freeze-drying. After this procedure a white, uncongested substance remained (Fig. D-1). The amount of substance depended primarily on the dialyser type and on the eluent. Generally, 17.2% ethanol eluates contained more leaching substances than ddH_2O eluates.

Fig. D-1 Lyophilised eluate (F60S, 17.2% EtOH, 24 h) in a 250 ml flask

When dissolving the extracted substance in ≈ 4 ml pure eluent a viscous, yellowish eluate liquid emerged from the 17.2% ethanol eluates. The dissolving of ddH_2O eluates resulted in clear, less viscous liquids. Those liquids were used for the analysis and testing described below.

2 HPLC-MS/MS Analysis of Eluates

2.1 Total Ion Scan

In order to gather a general idea of substances present in the concentrated eluates, total ion scans of 5 eluates were performed. Those eluates were obtained by 4 or 24 h extraction from FX60 dialysers using ddH_2O or 17.2% EtOH as eluent. Those total ions scans were compared to the ones of pure eluents which served as control.

An exemplary figure of a chromatogram is shown in Fig. D-2. The HPLC-grade ddH_2O is depicted by a red line and an exemplary eluate by a blue line. When looking at these graphs it is apparent that a variety of ions was detectable even in purest water. After water was pumped through a FX60 dialyser for 4 h, there were distinct changes in the ion spectra (blue line). Especially palpable are the 2 additional peaks at time point 1.18 and 1.71. However, the mass to charge values (m/z) of each peak were extremely multifarious and could not be attributed to a single substance. One exemplary mass spectrum is given in Fig. D-3. It shows all m/z values which are derived from one single peak (elution time 1.18 min) of the full range scan shown in Fig. D-2.

The variety of ions detectable by the total ion scan impeded the qualification of single substances. Consequently, further analysis had to be focused on specific substances, which could be expected in the eluates.

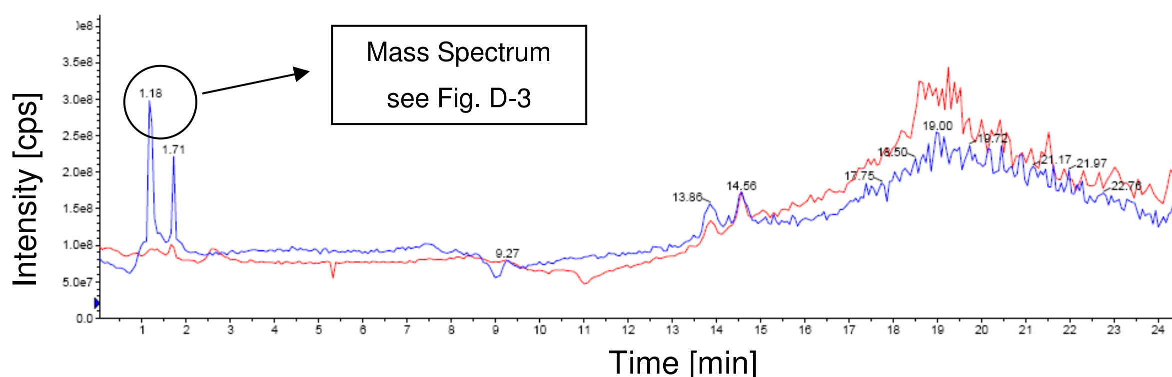


Fig. D-2 Chromatograms of full range scans of ddH_2O (red) und an exemplary eluate (FX60, ddH_2O, 4 h; blue). The flow of eluent through the assembled unit caused distinct changes in the chromatograph pattern.

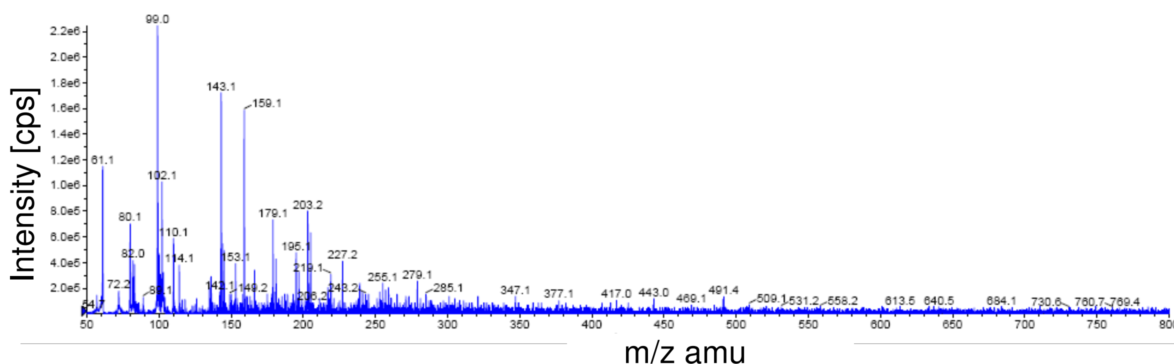


Fig. D-3 Exemplary mass spectrum of an eluate (FX60, ddH_2O, 4 h) at time 1.18 min

2.2 *Di(2-ethylhexyl)phthalate Analysis*

One of the substances expected to be found in the assembled circuit was DEHP. The DEHP content of eluates obtained from blood circuits containing PVC tubing was analysed by HPLC-MS/MS. As the dialysers used (FX60 and F80S) did not contain DEHP, any detected DEHP would have been leaching from the connecting tubings.

An exemplary chromatogram and corresponding mass spectrum of the DEHP analysis is shown in Fig. D-4 and Fig. D-5. While the internal standard was verifiable in each analysis, the DEHP concentration of the eluates did not reach the limit of detection (20 pg/ml) (Tab. D-1). Therefore the amounts of DEHP leaching from PVC tubings can be regarded as minimal and additional experiments using polyolefine tubing (DEHP free) were abandoned.

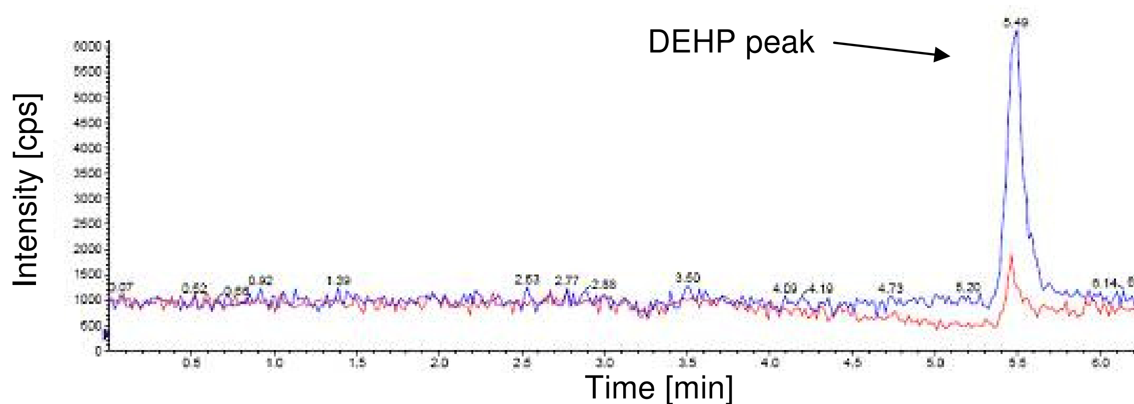


Fig. D-4 Chromatogram overlay of an exemplary eluate (F60S, 17.2% EtOH, 24 h) (red) and 100 pg/µl DEHP standard (blue).

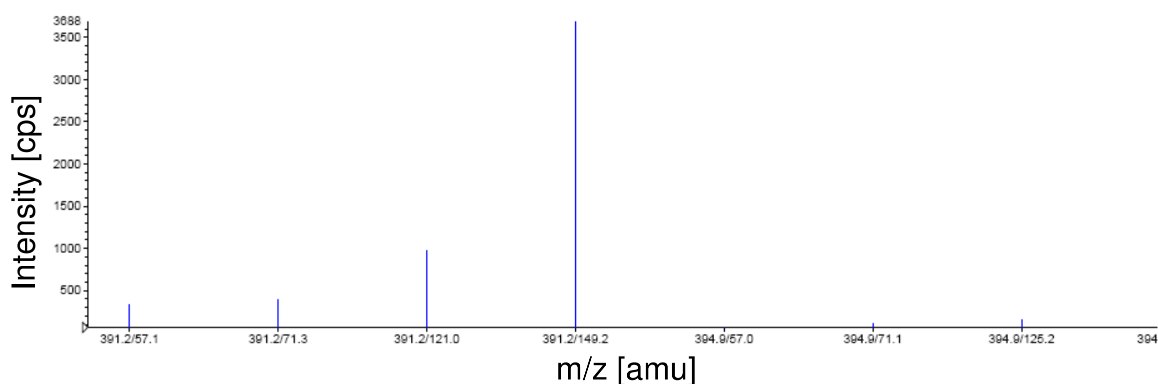


Fig. D-5 Mass spectrum of 100 pg/µl DEHP standard

Dialyser	Tubing	Eluent	Elution period	DEHP concentration per dialyser	Number of independent repetitions
F60S	PVC	ddH_2O	4 h	< d.l.	4
			24 h	< d.l.	6
		17.2 % EtOH	4 h	< d.l.	4
			24 h	< d.l.	4
FX60	PVC	ddH_2O	4 h	< d.l.	2
			24 h	< d.l.	4
		17.2 % EtOH	24 h	< d.l.	4

Tab. D-1 Elution modalities for DEHP measurement and results;
d.l. = detection limit

2.3 Bisphenol A Analysis

The BPA content of eluates obtained from 5 different dialysers under various elution conditions was determined by HPLC-MS/MS analysis. An exemplary chromatogram of BPA analysis and the corresponding mass spectrum is shown in Fig. D-6 and Fig. D-7.

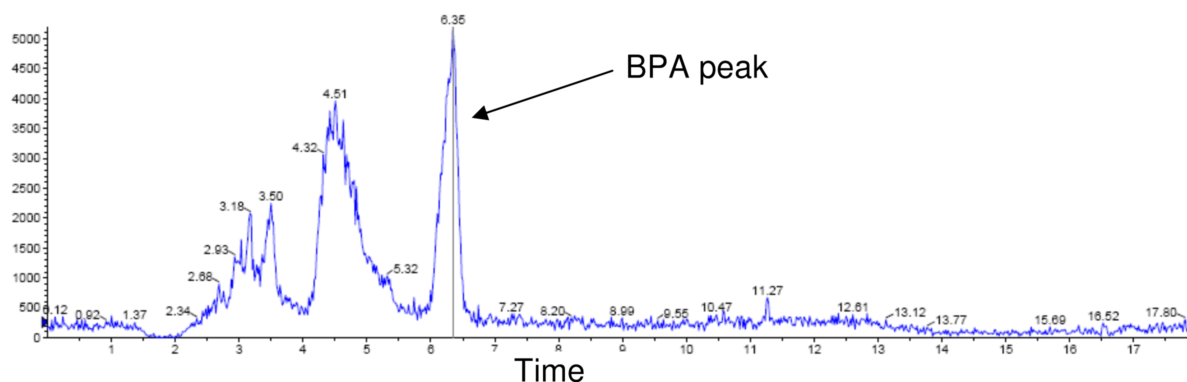


Fig. D-6 Chromatogram of an exemplary eluate (F60S, 24 h, 17.2% ethanol)

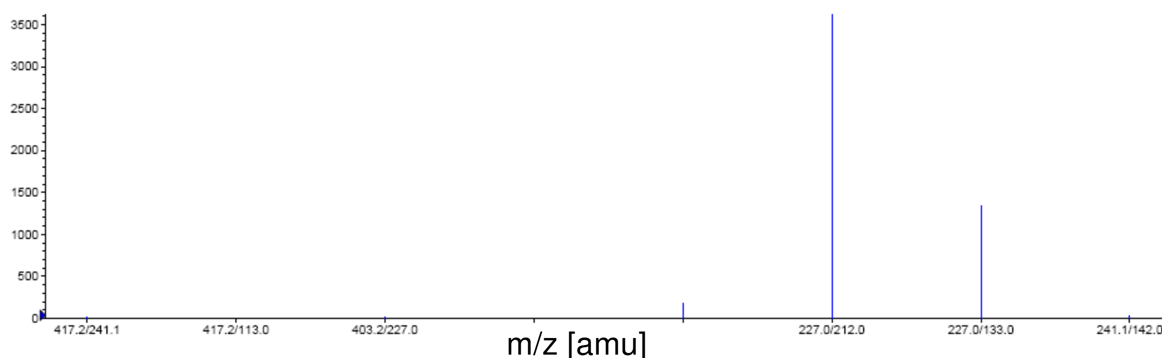


Fig. D-7 Mass spectrum of bisphenol A

BPA could be detected in each of the eluates. Generally, more BPA was detected in 17.2% EtOH eluates than in water eluates. The content ranged from 33.4 ng/dialyser (170H) to 2,321 ng/dialyser (FX80) when 17.2% ethanol was used as eluent and from 3.9 ng/dialyser (170H) to 44.8 ng/dialyser (F60S) when ddH_2O was used. Moreover the type of dialyser, the batches of the same dialyser (produced from different batches of polysolufone granulate) and the size of the membrane surface influenced the amount of leaching BPA. Most BPA could be extracted from FX80 dialysers and the least amount from 170H and FX60 dialysers. In most cases there was also more BPA detectable in the 24 h eluates than in the 4 h eluates. The quantity of BPA determined in the eluates is summarized in Tab. D-2.

Dialyser	Eluent	Time	[c] BPA [ng/dialyser]	S.D. [ng/dialyser]
FX60 Lot: LDV15104	ddH_2O	4 h	35.9	5.2
		24 h	39.8	3.9
	17.2% EtOH	24 h	49.6	1.5
FX60 Lot: NBV12101	ddH_2O	4 h	18.4	1.8
	17.2% EtOH	4 h	405.7	164.0
		24 h	482.7	89
F60S Lot: LCB29101	ddH_2O	4 h	38.0	3.6
		24 h	44.8	4.9
	17.2% EtOH	4 h	66.7	58.6
		24 h	89.3	13.4
FX80 Lot: NCX01130	ddH_2O	4 h	38.5	5.2
		24 h	37.2	7.2
	17.2% EtOH	4 h	1839.8	506.7
		24 h	2321.4	1074.9
FX80 Lot: NGV3021	17.2% EtOH	4 h	610.3	124.8
		24 h	681.6	69.8
HF80S Lot: NAK24160	ddH_2O	4 h	12.5	3.9
		24 h	17.7	1.9
HF80S Lot: NEK11140	17.2% EtOH	4 h	480.5	22.2
		24 h	719.8	19.3
170H Lot:S-4402-H-01	ddH_2O	4 h	3.9	0.11
		24 h	4.4	0.7
	17.2% EtOH	4 h	34	4.4
		24 h	33.4	11.9

Tab. D-2 Amount of BPA detected in different eluates. Values are given as the mean of measurement of at least 3 eluates.

In order to estimate the whole amount of BPA leaching from the dialysers it has to be considered that only 51% - 64% of the fluid filled into the dialyser could be regained. Most of the residual fluid remained in the hollow fibres of the dialysers due to capillary forces. Additionally a small part of the fluid remained in the dialysate compartment and could not be regained.

Presuming that BPA was uniformly dissolved in the whole fluid the total amount of leaching BPA was estimated (Tab. D-3). The maximum of leaching BPA was estimated to be 4.3 µg/dialyser.

Dialyser	Eluent	Time	Estimated amount of leaching BPA/dialyser [ng]
FX60 Lot: LDV15104	ddH_2O	4 h	66.5
		24 h	73.7
	17.2% EtOH	24 h	91.9
FX60 Lot: NBV12101	ddH_2O	4 h	34.1
	17.2% EtOH	4 h	751.3
		24 h	893.9
F60S Lot: LCB29101	ddH_2O	4 h	59.4
		24 h	70
	17.2% EtOH	4 h	104.2
		24 h	139.5
FX80 Lot: NCX01130	ddH_2O	4 h	71.3
		24 h	68.9
	17.2% EtOH	4 h	3407
		24 h	4298.9
FX80 Lot: NGV3021	17.2% EtOH	4 h	1130.2
		24 h	1262.2
HF80S Lot: NAK24160	ddH2O	4 h	18.7
		24 h	25.7
HF80S Lot: NEK11140	17.2% EtOH	4 h	717.2
		24 h	1074.3
170H Lot:S-4402-H-01	ddH_2O	4 h	6.4
		24 h	7.2
	17.2% EtOH	4 h	55.7
		24 h	54.8

Tab. D-3 Estimated amount of leaching BPA per dialyser

BPA could even be detected in pure eluent. This is probably due the ubiquitous presence of BPA, which can result in incidental contamination of the eluent - either during the manufacturing or bottling or during the subsequent handling process. However, even though the BPA concentration was above the limit of detection (0.56 ng/ml), it was below the limit of quantification (3.42 ng/ml). The amount of BPA leaching from standard PVC tubes (110 cm length) was also above the limit of detection but below the limit of quantification. Therefore the contribution of the tubing system to the total BPA content of our eluates is negligible.

3 Cytotoxicity Testing

In order to evaluate the cytotoxicity of eluates several test have been performed. Generally, 200 µl of the concentrated ddH_2O eluates or 100 µl of the concentrated 17.2% EtOH eluates were added to 5 ml cell culture media, leading to a 25 or 50-fold

dilution of the concentrated eluate. Due to the varying amounts of eluents obtained from the different dialysers, this led to the following concentration in media compared to the pure eluate (Tab. D-4):

Dialyser	Amount of eluate obtained	Amount of concentrated eluate	Concentration factor	Concentration factor of eluate in culture media compared to unprocessed eluate
FX60	125 ml	≈ 4 ml	31.25	1.25 (ddH_2O) / 0.625 (EtOH)
FX60S	200 ml	≈ 4 ml	50	2 (ddH_2O) / 1 (EtOH)
FX80	145 ml	≈ 4 ml	36.25	1.45 (ddH_2O) / 0.725 (EtOH)
HF80S	280 ml	≈ 4 ml	70	2.8 (ddH_2O) / 1.4 (EtOH)
170H	250 ml	≈ 4 ml	62.5	2.5 (ddH_2O) / 1.25 (EtOH)

Tab. D-4 Eluate concentrations in cell culture

Assuming a blood volume of 5 l the concentration of leaching substances in blood was 25 times (ddH_2O eluates) or 50 times (17.2% EtOH) lower than the ones reached in cell culture.

3.1 Cell Proliferation

First the simplest parameter of cytotoxicity – changes in cell proliferation – was assessed. The results of incubation of L5178Y cells with eluates of the 5 dialyser types tested are depicted in Fig. D-8 and Fig. D-9. The relative proliferation is presented in comparison to the vehicle control, which was normalized to 100% cell proliferation. The cell proliferation varied between 92% (HF80S, 4 h, ddH_2O) and 108% (FX80, 24 h, ddH_2O) of the vehicle control. Those variations were statistically not significant. Therefore it is obvious that neither 4 h eluates (Fig. D-8), nor 24 h eluates obtained with 17.2% EtOH or ddH_2O influenced the cell proliferation in comparison to the vehicle control. In contrast, incubation with the positive control MMS (50 µg/ml) reduced the cell proliferation significantly.

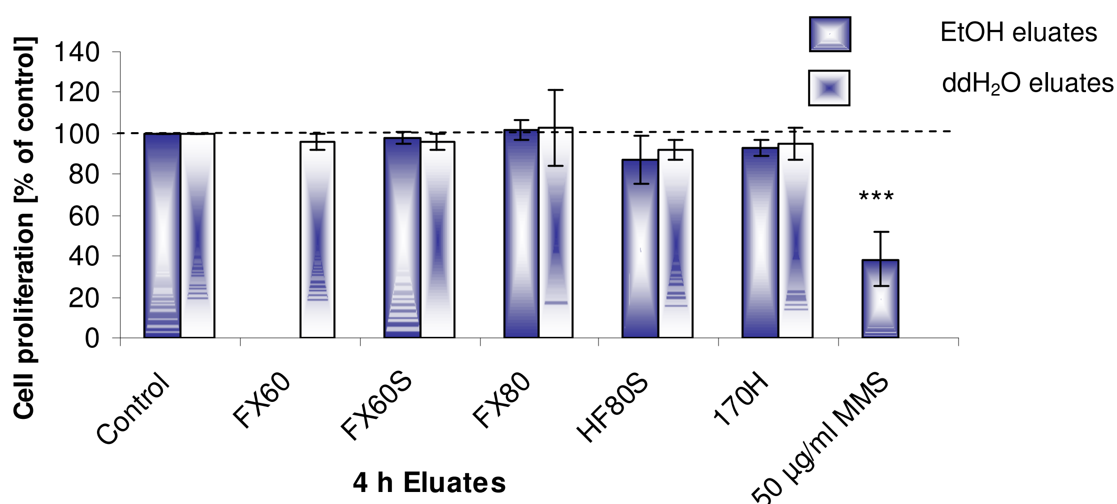


Fig. D-8 Relative cell proliferation of L5178Y cells after incubation with 4 h eluates of the dialysers FX60, FX60S, FX80, HF80S and 170H for 24 h. Methyl-methane-sulfonate (MMS; 50 µg/ml) served as positive control. Results are shown as mean ± S.D. of three independent experiments. *** $p \leq 0.001$

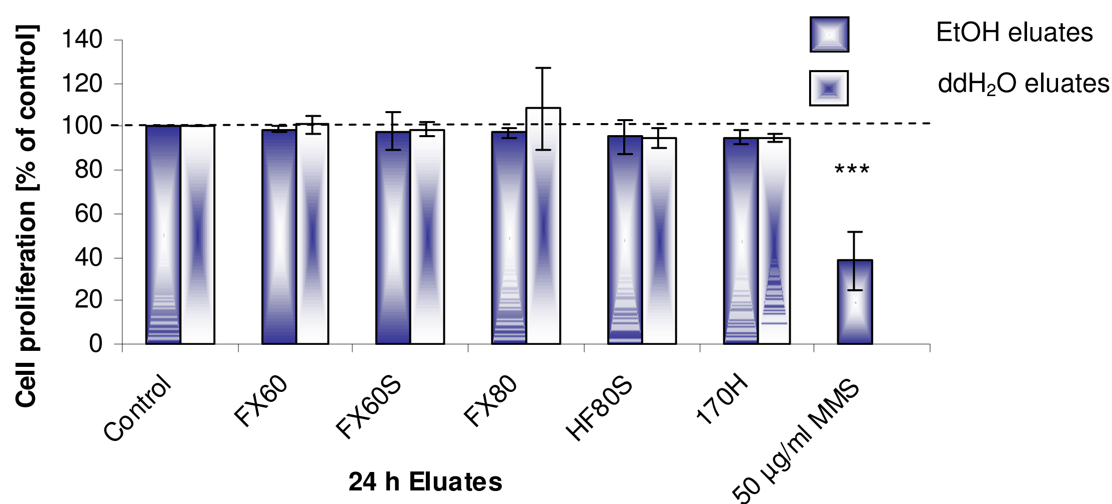


Fig. D-9 Relative cell proliferation of L5178Y cells after incubation with 24 h eluates of the dialysers FX60, FX60S, FX80, HF80S and 170H for 24 h. Methyl-methane-sulfonate (MMS; 50 µg/ml) served as positive control. Results are shown as mean ± S.D. of three independent experiments. *** $p \leq 0.001$

3.2 Mitosis Frequency

Second, the influence of eluates on the mitotic frequency of L5178Y cells was analysed. 24 h incubation with the eluates of varying types of dialysers did not alter the mitotic frequency of cells compared to the vehicle control - regardless of the elution conditions. Results of three independent experiments can be seen in Fig.

D-10 and Fig. D-11. Owing to the varying time spans needed for cell division of different cell passages, the mitotic frequency is presented as values relative to the control, not in absolute numbers. The mitotic frequency varied between a relative mitotic frequency of 0.8 (HF80S, 24 h, 17.2% EtOH) and 1.4 (FX80, 4 h, ddH_2O). These effects were statistically not significant.

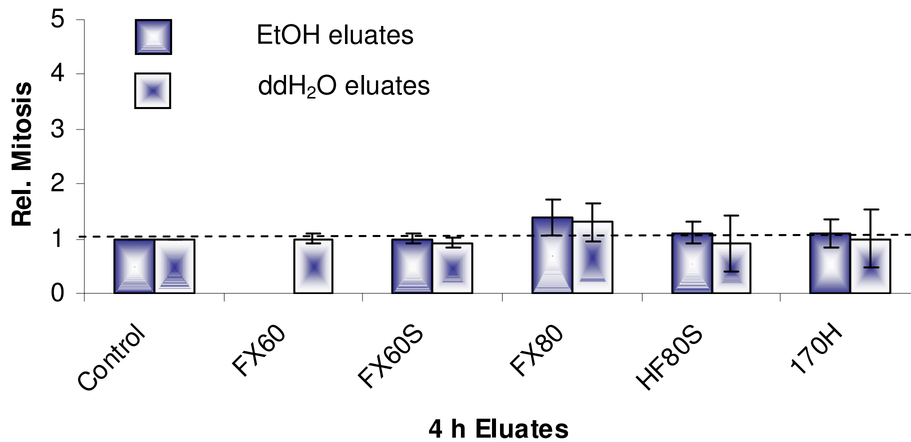


Fig. D-10 Relative mitosis frequency of L5178Y cells after incubation with 4 h eluates of the dialysers FX60, FX60S, FX80, HF80S and 170H for 24 h. Results are shown as mean ± S.D. of three independent experiments.

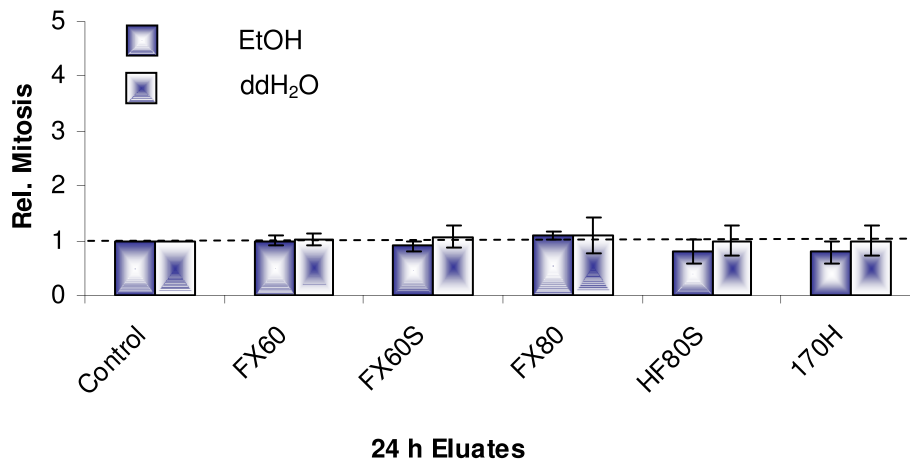


Fig. D-11 Relative mitosis frequency of L5178Y cells after incubation with 24 h eluates of the dialysers FX60, FX60S, FX80, HF80S and 170H for 24 h. Results are shown as mean ± S.D. of three independent experiments.

3.3 Apoptosis

Finally, it was analysed whether eluates are capable of inducing apoptosis. First, the amount of apoptotic cells after incubation with FX60 and F60S eluates was determined by annexin V staining and flow cytometry (Fig. D-12a, Fig. D-12b). 24 h incubation of L5178Y cells with those eluates did not induce apoptosis, while the positive control (100 µM $NaAsO_2$) was effective. Simultaneously, apoptosis induction was determined by microscopic analysis. The results obtained by flow cytometry and microscopy were identical (data not shown). Because the microscopic analysis of apoptosis could be performed along with analysis for mitosis and MN, the remaining eluates were analysed by microscopy only. The percentage of apoptotic cells in the vehicle control was 0.1 ± 0.05%. The percentage of apoptotic cells in the cells treated with eluate lay between 0 - 0.12% (Fig. D-13, Fig. D-14). Therefore it can be concluded that eluates did not induce apoptosis in L5178Y cells.

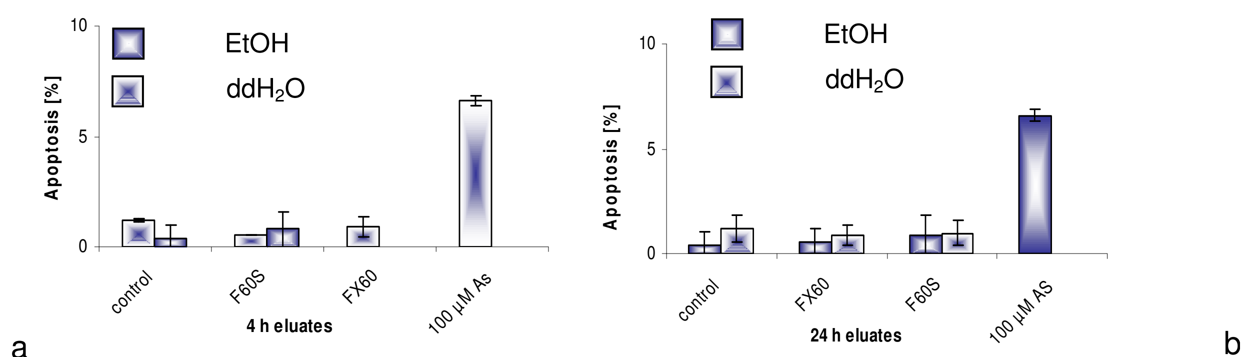


Fig. D-12 Percentage of apoptotic L5178Y cells after incubation with 4 h (a) and 24 h (b) of the dialysers F60S and FX60 for 24 h. The analysis was performed by flow cytometry. 100 µM. $NaAsO_2$ served as positive control. Results are shown as mean ± S.D. of three independent experiments.

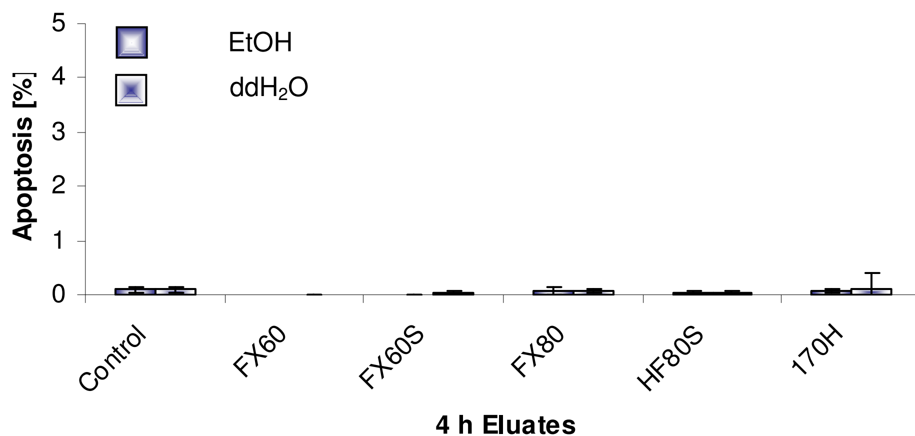


Fig. D-13 Percentage of apoptotic L5178Y cells after incubation with 4 h eluates of the dialysers FX60, FX60S, FX80, HF80S and 170H for 24 h. Results are shown as mean ± S.D. of three independent experiments.

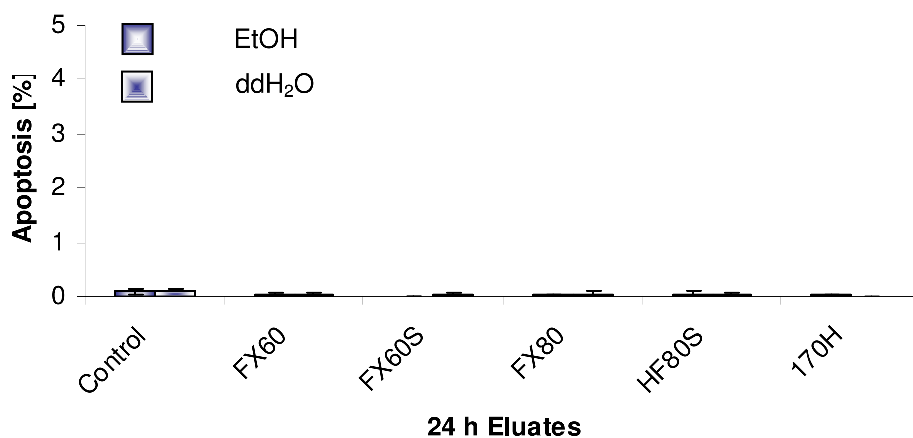


Fig. D-14 Percentage of apoptotic L5178Y cells after incubation with 24 h eluates of the dialysers FX60, FX60S, FX80, HF80S and 170H for 24 h. Results are shown as mean ± S.D. of three independent experiments.

In summary, none of the eluates exhibited any cytotoxic effect in L5178Y cells.

4 Genotoxicity Tests

After the cytotoxicity tests revealed no evidence for cytotoxicity caused by eluates the potential genotoxicity of the eluates was analysed by micronucleus test and comet assay.

4.1 Micronucleus Frequency

As described earlier, incubation with eluates did not influence cell proliferation, therefore the use of Cyt B in the MN assay was not necessary. None of the 20 eluates tested increased the micronucleus frequency compared to vehicle control values, while the positive control MMC (0.13 µg/ml) raised the number of micronucleic cells considerably (Fig. D-15 and Fig. D-16). The relative amount of MN in the control was normalized to 1. The values of cells treated with eluate varied between 0.8 (170H, 4 h, ddH_2O) and 2.2 (FX80, 4 h, 17.2% EtOH) ± 1. This range was within the 95% confidence interval of the vehicle control and therefore statistically not significant.

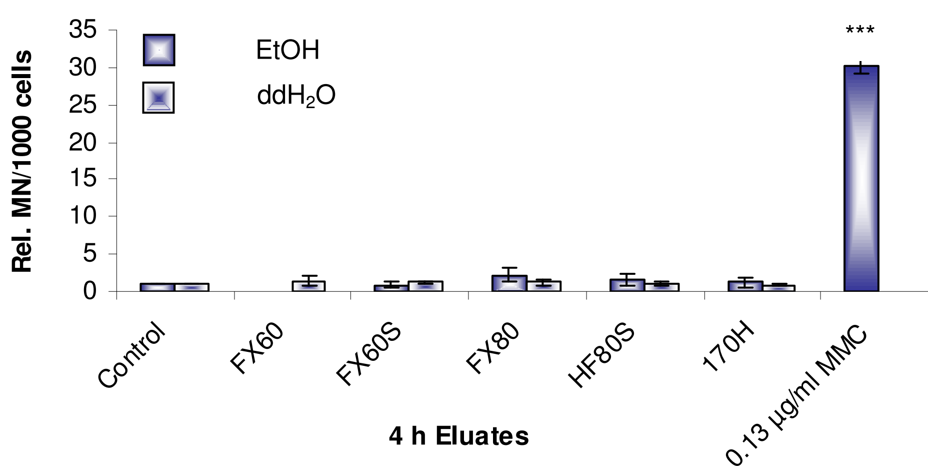


Fig. D-15 Relative number of micronuclei per thousand L5178Y cells after incubation with 4 h eluates of the dialysers FX60, FX60S, FX80, HF80S and 170H for 24 h. Mitomycin C (MMC; 0.13 µg/ml) served as positive control. Results are shown as mean ± S.D. of three independent experiments. *** $p \leq 0.001$

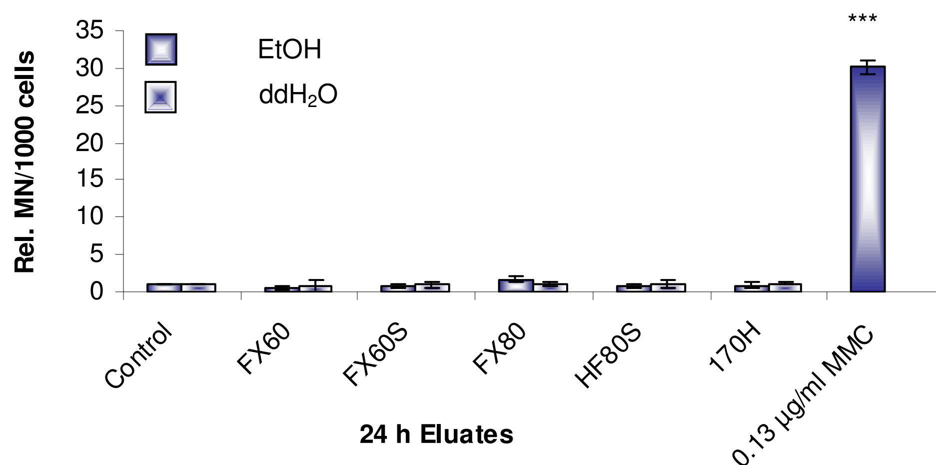


Fig. D-16 Relative number of micronuclei per thousand L5178Y cells after incubation with 24 h eluates of the dialysers FX60, FX60S, FX80, HF80S and 170H for 24 h. Mitomycin C (MMC; 0.13 µg/ml) served as positive control. Results are shown as mean ± S.D. of three independent experiments. *** $p \leq 0.001$

4.2 Comet Assay

The second genotoxicity test was the comet assay. Incubation of L5178Y cells with eluates of the dialysers FX60, FX60S, FX80, HF80S or 170H did not increase the percentage of DNA in tail compared to the ones in the control to a statistically significant degree (Fig. D-17 and Fig. D-18). The relative amount of DNA in tail varied between 0.8 (HF80S, 4 h, 17.2% EtOH) and 1.9 (FX60S, 24 h, ddH_2O) compared to 1 in the vehicle control. The was a slight tendency for increased genomic damage after incubation with 17.2% EtOH eluates of FX60 and F60S dialysers; however, statistical significance was not reached. As expected the positive control MMS (50 µg/ml) increased the amount of DNA in tail considerably.

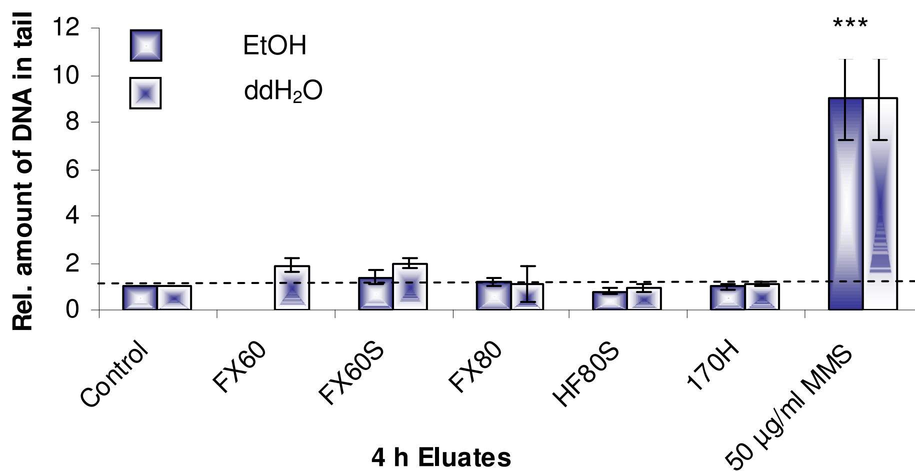


Fig. D-17 Relative DNA damage of L5178Y cells detected by the comet assay after incubation with 4 h eluates of the dialysers FX60, FX60S, FX80, HF80S and 170H for 24 h. Methyl-methane-sulfonate (MMS; 50 µg/ml) served as positive control. Results are shown as mean ± S.D. of three independent experiments. *** $p \leq 0.001$

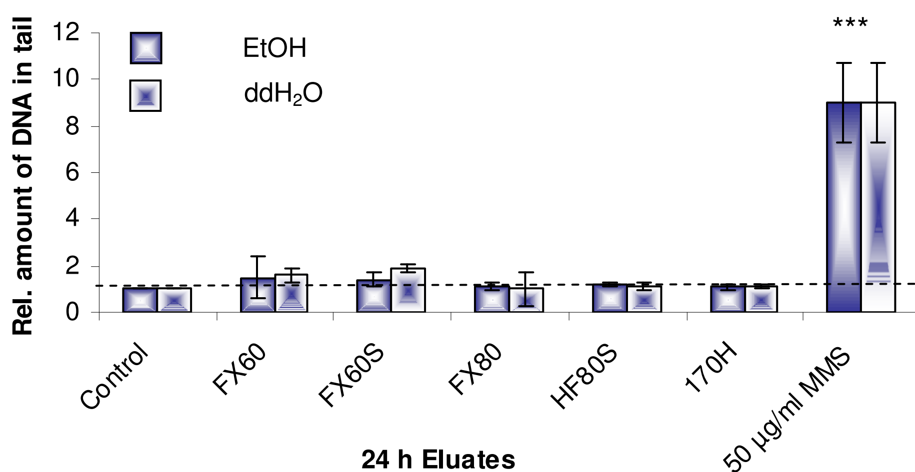


Fig. D-18 Relative DNA damage of L5178Y cells detected by the comet assay after incubation with 24 h eluates of the dialysers FX60, FX60S, FX80, HF80S and 170H for 24 h. Methyl-methane-sulfonate (MMS; 50 µg/ml) served as positive control. Results are shown as mean ± S.D. of three independent experiments. *** $p \leq 0.001$

4.3 Test for Estrogenic Activity

Because BPA possesses estrogenic activity and it can be detected in dialyser eluates, tests for estrogenic activity (E-screens) were performed. The E-screens were conducted with MCF-7 cells which are more sensitive towards solvents in media than L5178Y cells. Therefore only 50 µl of 17.2% EtOH eluates or 100 µl of ddH_2O eluates

could be added. This led to a 50% decrease of eluate concentration in the media compared to the toxicity testing.

4.3.1 E-Screen

Incubation of MCF-7 cells with 17.2% EtOH eluates of the dialysers FX60, FX60S, FX80, HF80S and 170H (24 h eluates only) increased the proliferation by 30% - 60%. Incubation with ddH_2O eluates of the dialysers FX60, FX60S, FX80, HF80S and 170H (24 h eluates) increased the proliferation slightly, but not in a statistically significant way (Fig. D-19).

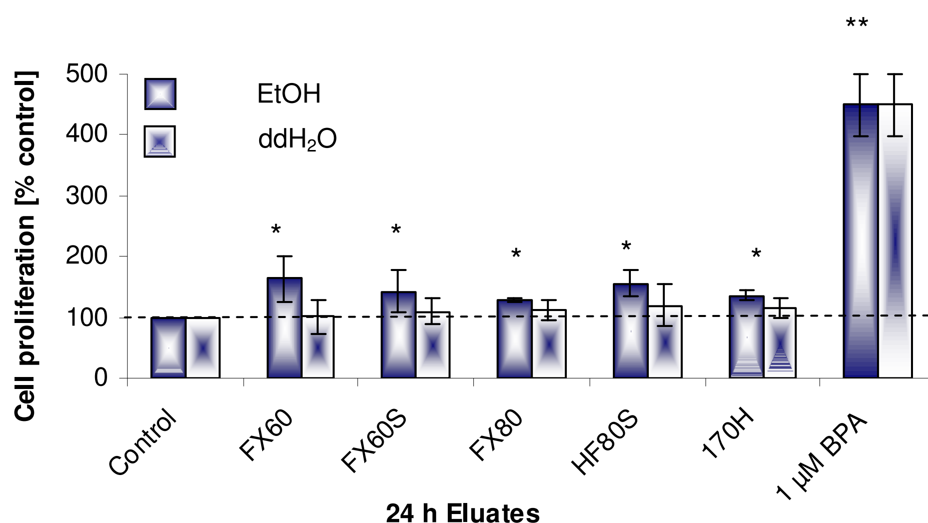


Fig. D-19 Relative proliferation of MCF-7 cells after 8 days incubation with 24 h eluates of the dialysers FX60, FX60S, FX80, HF80S and 170H. Bisphenol A (1µM) served as positive control. Results are shown as mean ± S.D. of three independent experiments. * $p \leq 0.05$; ** $p \leq 0.01$, *** $p \leq 0.001$

In order to decide whether the increase of proliferation was due to BPA in the eluates, a calibration curve of pure BPA in the range of 0.1 - 1000 nM was established (Fig. D-20). The proliferation of MCF-7 cells started to increase significantly after incubation with 31.6 nM BPA. This equals 7.2 ng/ml BPA. This increase of proliferation was dose-dependent.

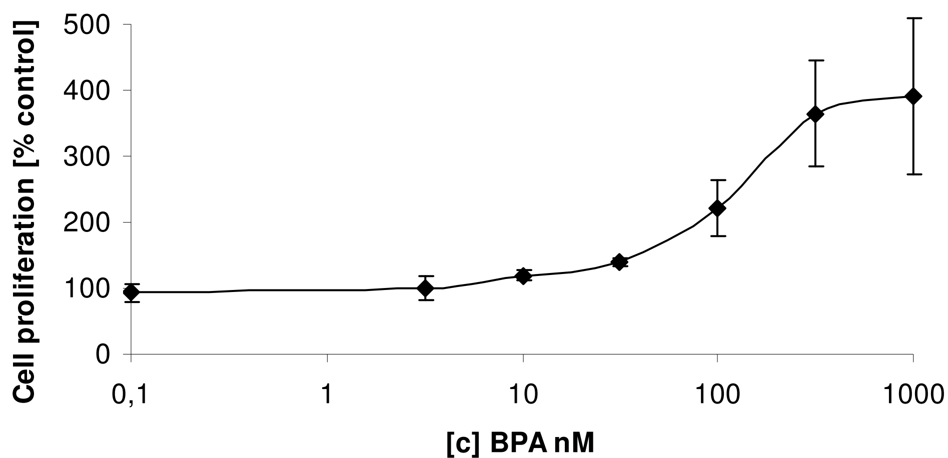


Fig. D-20 Relative proliferation of MCF-7 cells after incubation with bisphenol A for 8 days. Results are shown as mean ± S.D. of three independent experiments.

5 Summary of the Toxicity Testing

To summarize: in the range of concentration tested eluates did not cause any effect in the cytotoxicity and genotoxicity test of this study (Tab. D-5). The only effect was an increase of proliferation of estrogen sensitive MCF-7 cells by 17.2% ethanol eluates.

Test system	Eluent	Time	FX60	F60S	FX80	HF80S	170H
Proliferation	ddH_2O	4 h	-	-	-	-	-
		24 h	-	-	-	-	-
	EtOH	4 h	-	-	-	-	-
		24 h	-	-	-	-	-
Mitosis frequency	ddH_2O	4 h	-	-	-	-	-
		24 h	-	-	-	-	-
	EtOH	4 h	-	-	-	-	-
		24 h	-	-	-	-	-
Apoptosis	ddH_2O	4 h	-	-	-	-	-
		24 h	-	-	-	-	-
	EtOH	4 h	-	-	-	-	-
		24 h	-	-	-	-	-
Micronucleus frequency	ddH_2O	4 h	-	-	-	-	-
		24 h	-	-	-	-	-
	EtOH	4 h	-	-	-	-	-
		24 h	-	-	-	-	-
Comet-Assay	ddH_2O	4 h	-	-	-	-	-
		24 h	-	-	-	-	-
	EtOH	4 h	-	-	-	-	-
		24 h	-	-	-	-	-
E-Screen	ddH_2O	24 h	-	-	-	-	-
	EtOH	24 h	+	+	+	+	+

Tab. D-5 Summary of the toxicity testing.

6 Uremic Toxins

Several uremic toxins with suspected genotoxicity have been analysed in this study: Hcy and its derivate Hcy-T as well as leptin and AGEs.

6.1 Homocysteine and Homocysteine-Thiolactone

6.1.1 Cytotoxicity Testing

The uremic toxin studied most intensively was Hcy. Two cell lines were chosen for the analysis: L5178Y cells and HL60 cells. L5178Y cells were selected because they are an established cell line for genotoxicity testing. HL60 cells were used because they are known to be sensitive towards oxidative stress, an expected effect

of Hcy. Toxicity tests of the Hcy derivate Hcy-T were conducted with L5178Y cells only.

6.1.1.1 Proliferation

In order to determine the concentration at which Hcy displays cytotoxicity in L5178Y cells range-finding experiments were performed. The Hcy concentrations varied from 0.1 mM to 10 mM. Two incubation times were chosen: 24 h and 120 h, the latter should resemble long term exposure which is relevant for dialysis patients. Starting at 3.16 mM Hcy 120 h incubation caused a considerable reduction of proliferation (Fig. D-21). The same effects could be observed after 24 h of exposure, but only at concentrations higher than 3.16 mM.

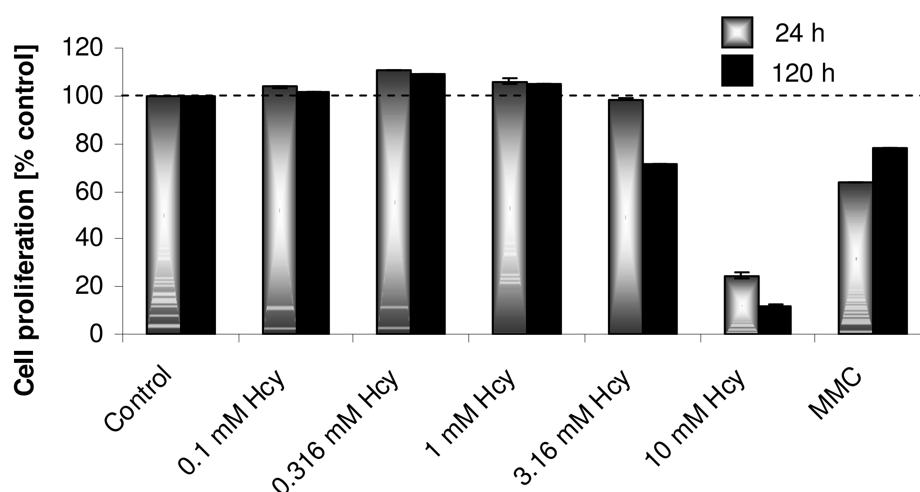


Fig. D-21 Proliferation of L5178Y cells after incubation with homocysteine for 24 h (lighter bars) or 120 h (black bars), respectively. Mitomycin C (MMC; 125 ng/ml) served as positive control. (Single experiment)

Subsequently, improved cytotoxicity tests were conducted with L5178Y and HL60 cells. A 24 h incubation with 4 mM Hcy decreased cell proliferation of L5178Y cells to a statistically significant degree. Based on their longer cell cycle HL60 cells were incubated for 48 h. The proliferation of HL60 cells decreased slightly but not significantly up to concentrations of 5 mM (Fig. D-22).

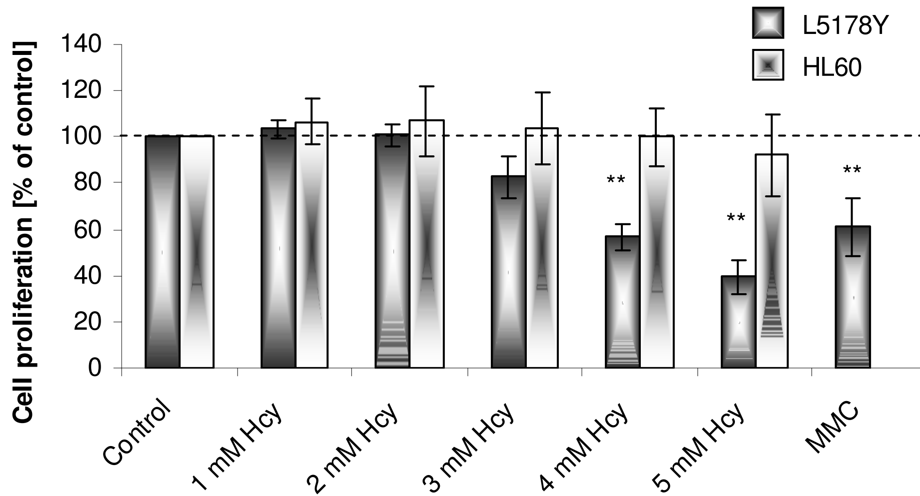


Fig. D-22 Proliferation of L5178Y cells (dark bars) and HL60 cells (bright bars) relative to the vehicle control after incubation with homocysteine for 24 h (L5178Y) or 48 h (HL60) respectively. Mitomycin C (MMC; 125 ng/ml) served as positive control. Results are shown as mean ± S.D. of three independent experiments * $p \le 0.05$, ** $p \le 0.01$, *** $p \le 0.001$

Hcy-T was more cytotoxic than Hcy. A statistical significant reduction of proliferation could already be observed at 1 mM Hcy-T already (Fig. D-23).

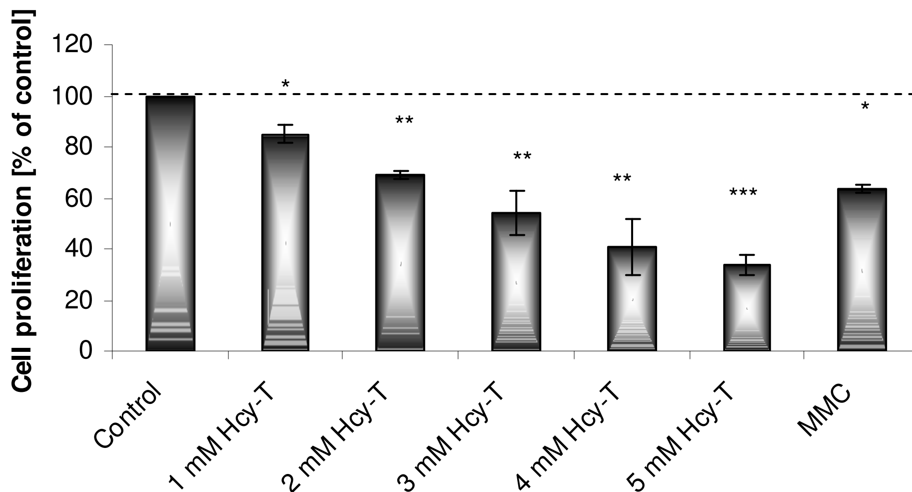


Fig. D-23 Proliferation of L5178Y cells relative to the vehicle control after incubation with homocysteine-thiolactone (Hcy-T) for 24 h. Mitomycin C (MMC; 125 ng/ml) served as positive control. Results are shown as mean ± S.D. of three independent experiments * $p \le 0.05$, ** $p \le 0.01$, *** $p \le 0.001$

6.1.1.2 Mitosis

In parallel to cell proliferation the number of mitotic cells was analysed microscopically. The mitotic frequency of L5178Y cells decreased statistically significant way at 5 mM Hcy, a concentration which was also cytotoxic. In HL60 cells a tendency towards a reduced percentage of mitotic cells could be observed at 5 mM Hcy (Fig. D-24). However this tendency was statistically non-significant.

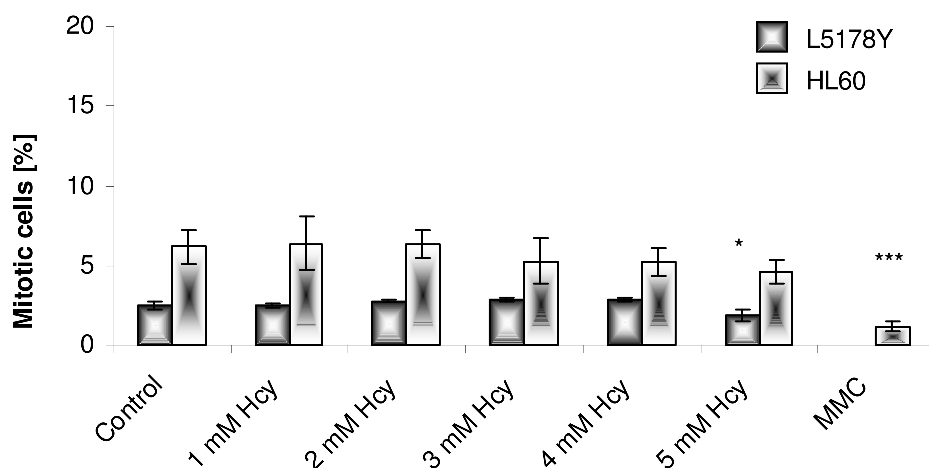


Fig. D-24 Percentage of cells undergoing mitosis in L5178Y cells (dark bars) and HL60 cells (bright bars) after incubation with homocysteine for 24 h (L5178Y) or 48 h (HL60) respectively. Mitomycin C (MMC; 125 ng/ml) served as positive control. Results are shown as mean ± S.D. of three independent experiments * $p \le 0.05$, ** $p \le 0.01$, *** $p \le 0.001$

6.1.1.3 Apoptosis

In addition, the percentage of apoptotic cells was determined microscopically. Hcy did not induce apoptosis in L5178Y cells up to concentrations of 5 mM, whereas it induced apoptosis in HL60 cells (Fig. D-25). This effect was statistically significant at concentrations of 4 mM Hcy and higher. At 5 mM the number of apoptotic cells was roughly tripled compared to the vehicle control.

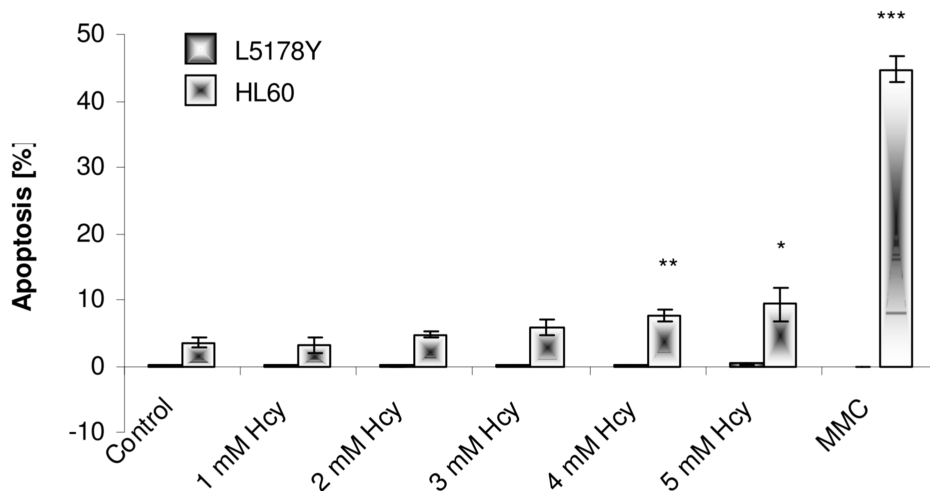


Fig. D-25 Percentage of cells undergoing apoptosis in L5178Y cells (dark bars) and HL60 cells (light bars) after incubation with homocysteine for 24 h (L5178Y) or 48 h (HL60) respectively. Mitomycin C (MMC; 125 ng/ml) served as positive control. Results are shown as mean ± S.D. of three independent experiments * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$

The possible pro-apoptotic effects of Hcy-T were only analysed in L5178Y cells. A stitistically significant and dose-dependent induction of apoptosis was observed at 3 mM - 5mM Hcy-T (Fig. D-26).

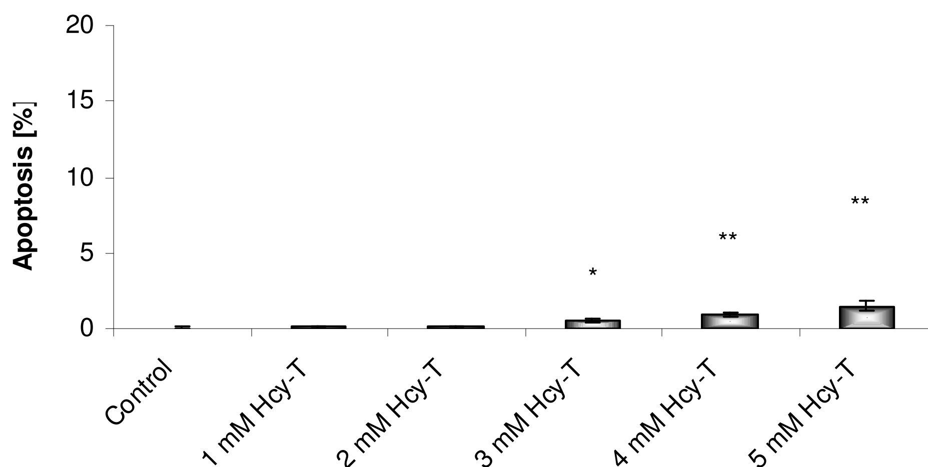


Fig. D-26 Percentage of cells undergoing apoptosis in L5178Y cells after incubation with homocysteine-thiolactone (Hcy-T) for 24 h respectively. Mitomycin C (MMC; 125 ng/ml) served as positive control. Results are shown as mean ± S.D. of three independent experiments * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$

6.1.2 Genotoxicity Tests

6.1.2.1 Micronucleus Assay

Simultaneously to the range-finding experiments for cytotoxicity the range-finding experiments for genotoxicity were conducted. The micronucleus frequency started to increase after incubation with 3.16 mM Hcy for 24 h (Fig. D-27). Incubation for 5 days did increase the number of micronuclei even further but did not lower the threshold.

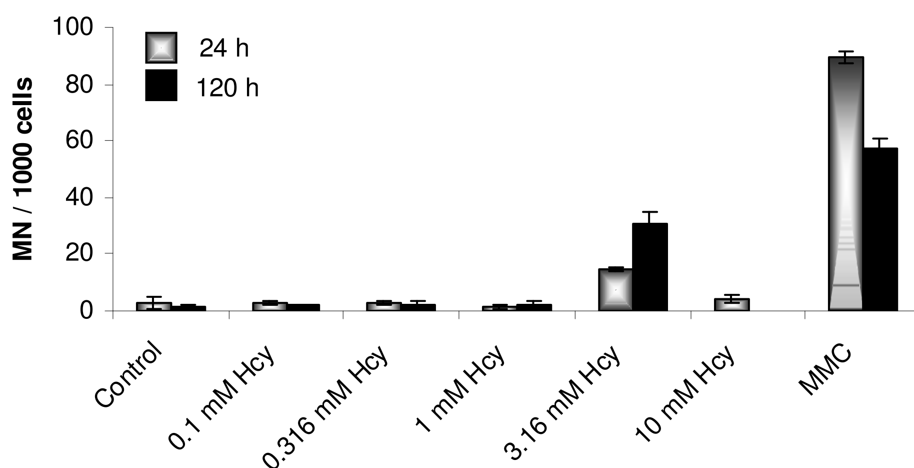


Fig. D-27 Micronucleus induction in L5178Y cells after incubation with homocysteine for 24 h (brighter bars) or 120 h (black bars) respectively. Mitomycin C (MMC; 125 ng/ml) served as positive control.

Subsequently, improved micronucleus assays showed a significant increase of MN after 24 h incubation of L5178Y with 3 mM Hcy. This effect was even more pronounced at higher concentrations (Fig. D-28).

In HL60 cells a significant increase of the micronucleus frequency could only be observed at 3 mM Hcy, not at higher or lower concentrations. As there was no dose-dependency a coincidental finding cannot be ruled out.

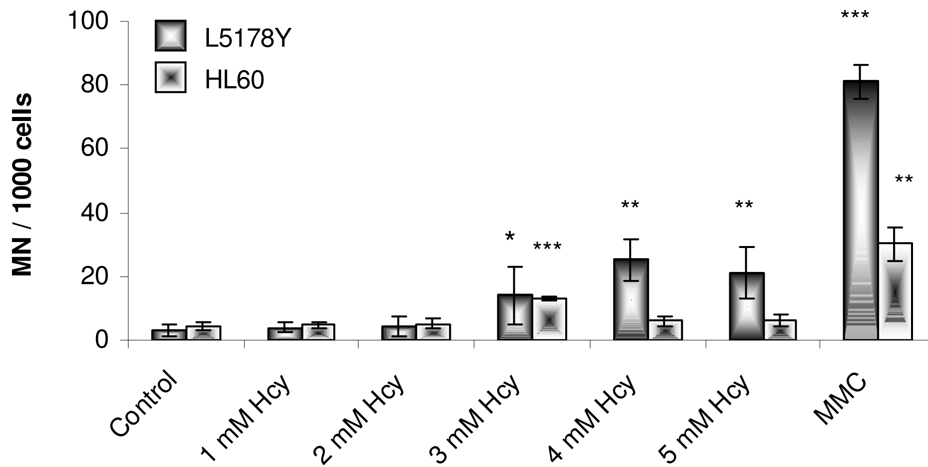


Fig. D-28 Micronucleus induction in L5178Y cells (dark bars) and HL60 cells (bright bars) after incubation with homocysteine for 24 h (L5178Y) or 48 h (HL60) respectively. Mitomycin C (MMC; 125 ng/ml) served as positive control. Results are shown as mean ± S.D. of three independent experiments * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$

Apart from the induction of MN, Hcy also reduced the mitotic rate of L5178Y cells, therefore additional MN assays with Cyt B were performed. However, the results did not differ from the MN tests described above.

The Hcy derivate Hcy-T elevated the MN frequency at 1 mM, the same concentration which reduced cell proliferation. The number of MN increased in a dose-dependent manner (Fig. D-29).

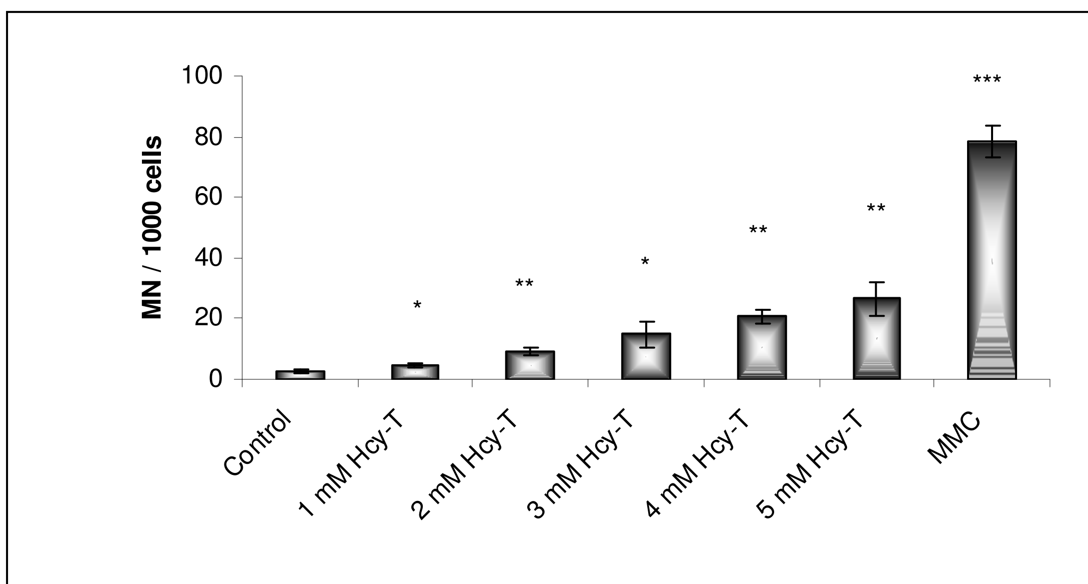


Fig. D-29 Micronucleus induction in L5178Y cells after incubation with homocysteine-thiolactone (Hcy-T) for 24 h. Mitomycin C (MMC; 125 ng/ml) served as positive control

To evaluate whether MN induction is cell-type specific for L5178Y cells, MN tests with additional cell lines (CaCo, TK6 and LLC-PK1) were performed (Fig. D-30). Hcy and Hcy-T increased the MN frequency at 4 mM, the same range at which it induced MN in L5178Y. Therefore the genotoxicity seems to occur in a broad range of cell lines.

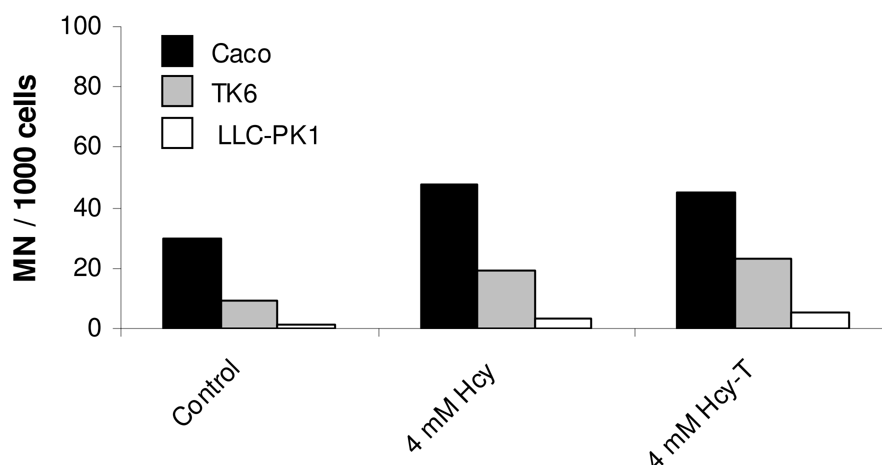


Fig. D-30 Micronucleus induction in CaCo cells (black bars) TK6 cells (grey bars) and LLC-PK1 cells after incubation with homocysteine and homocysteine-thiolactone (Hcy-T) for 48 h.

6.1.2.2 Comet Assay

While the MN frequency test yielded evidence for genotoxicity of Hcy and Hcy-T, the second genotoxicity test, the comet assay, did not support this finding. Hcy did not increase the relative amount of DNA in tail of L5178Y or HL60 cells up to cytotoxic concentrations (Fig. D-31); the same was true for Hcy-T (Fig. D-32).

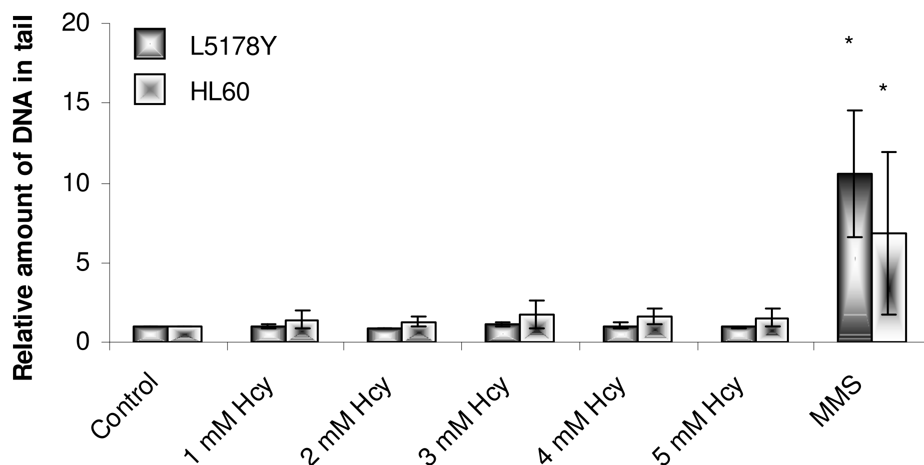


Fig. D-31 DNA damage analysed by comet assay (relative percentage of DNA in tail) after incubation of L5178Y cells (dark bars) and HL60 cells (bright bars) with homocysteine for 24 h. Methyl-methane-sulfonate (MMS; 50 µM) served as positive control. Results are shown as mean ± S.D. of three independent experiments * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$

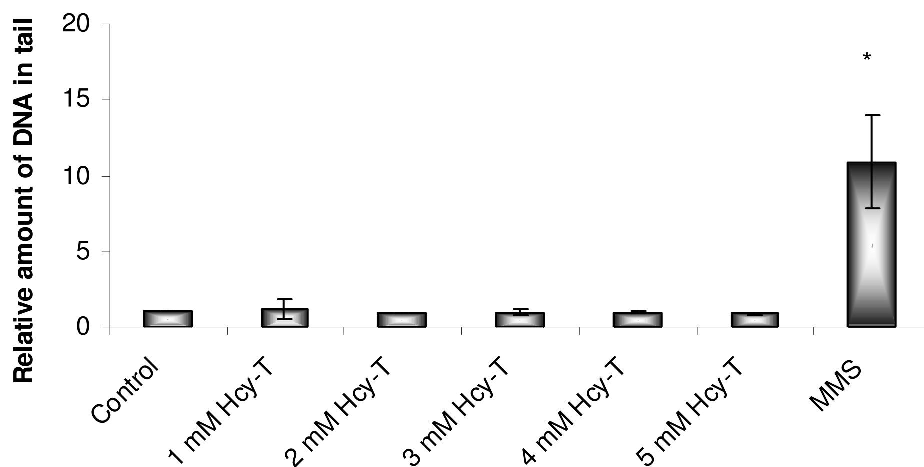


Fig. D-32 DNA damage analysed by comet assay (relative percentage of DNA in tail) after incubation of L5178Y cells with homocysteine-thiolactone (Hcy-T) for 24 h. Methyl-methane-sulfonate (MMS; 50 µM) served as positive control. Results are shown as mean ± S.D. of three independent experiments * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$

DNA damage, like DNA strand breaks, is normally detectable by comet assay. However, DNA strand breaks can be repaired by enzymes and may therefore be overlooked in a 24 h assay. In order to rule out this possibility, short-term comet assays (incubation period 2-24 h) were conducted on L5178Y cells. No increased

DNA damage could be observed after incubation with Hcy (Fig. D-33) or Hcy-T (Fig. D-34).

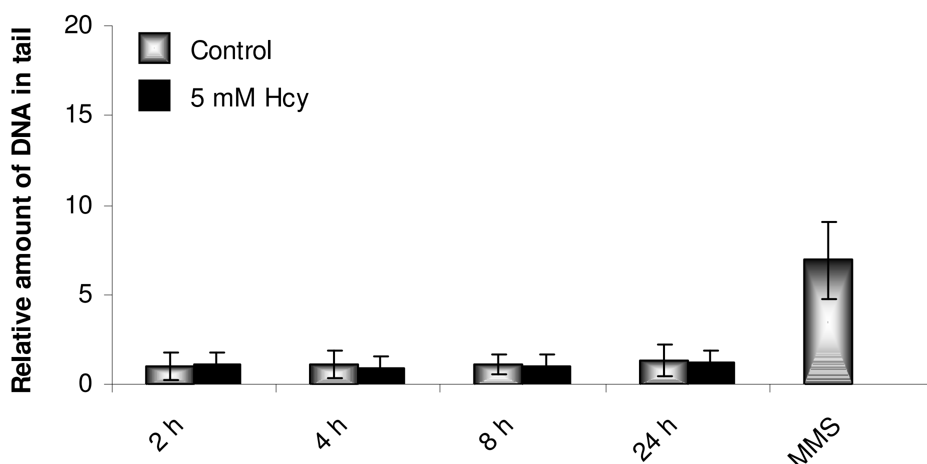


Fig. D-33 DNA damage analysed by comet assay (relative percentage of DNA in tail) after incubation of L5178Y cells with 5 mM homocysteine for 2 - 24 h. Methyl-methane-sulfonate (MMS; 50 µM) served as positive control (single experiment).

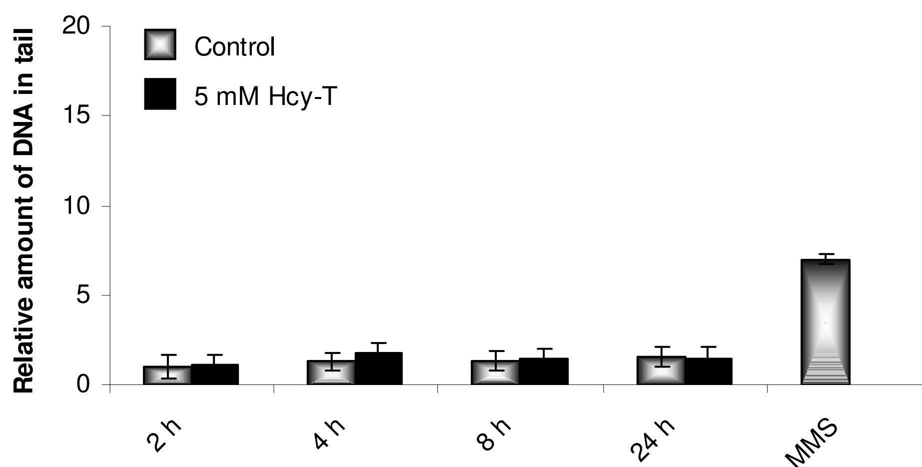


Fig. D-34 DNA damage analysed by comet assay (relative percentage of DNA in tail) after incubation of L5178Y cells with 5 mM homocysteine-thiolactone (Hcy-T) for 2 - 24 h. Methyl-methane-sulfonate (MMS; 50 µM) served as positive control (single experiment).

6.1.3 Oxidative Stress

6.1.3.1 Oxidative Stress & Micronuclei

One possible reason for increased genomic damage is oxidative stress. In order to analyse whether Hcy induces MN by causing oxidative stress, the radical scavenger N-acetylcysteine (NAC) was added to cells challenged by Hcy. Addition of NAC did not reduce the MN frequency of L5178Y cells induced by Hcy or Hcy-T (Fig. D-35).

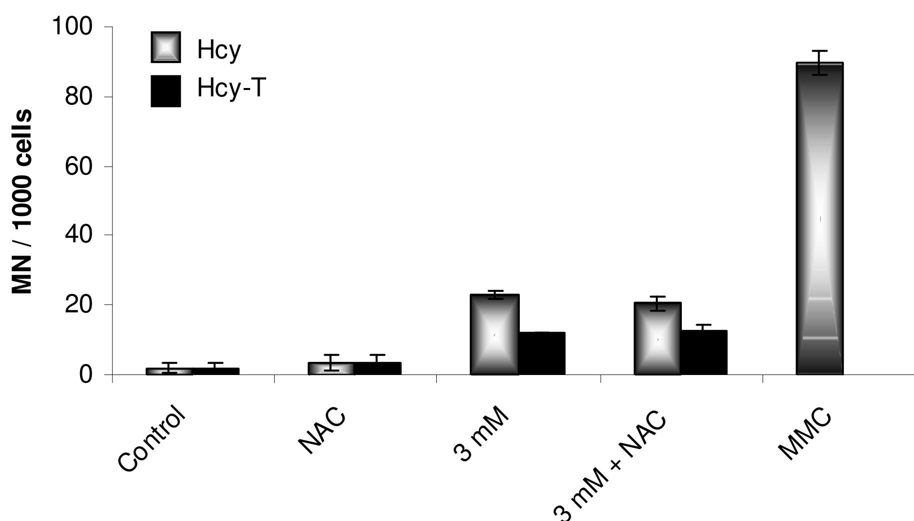


Fig. D-35 Micronucleus frequency found in L5178Y cells after incubation with 3 mM homocysteine or homocysteine-thiolactone (Hcy-T) and N-acetylcysteine for 24 h. (single experiment)

6.1.3.2 Flow Cytometric Analysis of Oxidative Stress

In order to determine whether Hcy induces oxidative stress at all, flow cytometric analyses of HL60 cells after incubation with 3 mM Hcy were conducted. 0.5 mM H_2O_2 served as positive control. To analyse whether Hcy can modulate oxidative stress, experiments of co-incubation with H_2O_2 and Hcy were performed. Oxidative stress can be very short-lived, therefore incubation periods from 30 min to 24 h were analysed. During this period Hcy did not induce oxidative stress observable by DCF fluorescence. On the contrary: Hcy was able to reduce oxidative stress induced by H_2O_2 (Fig. D-36, Fig. D-38).

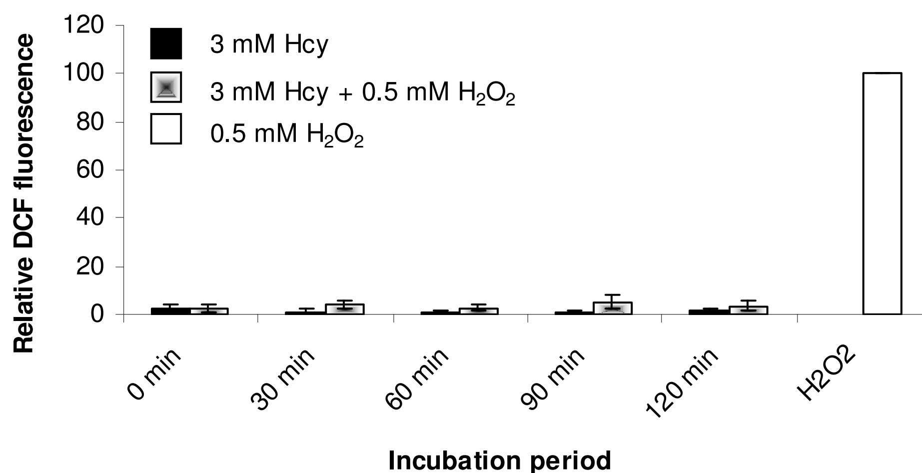


Fig. D-36 Oxidative stress level measured by relative DCF fluorescence after 30 to 120 min incubation of HL60 cells with 3 mM homocysteine (black bars) or pre-treated with homocysteine and challenged with H_2O_2 (grey bars). Results are shown as mean ± S.D. of three independent experiments.

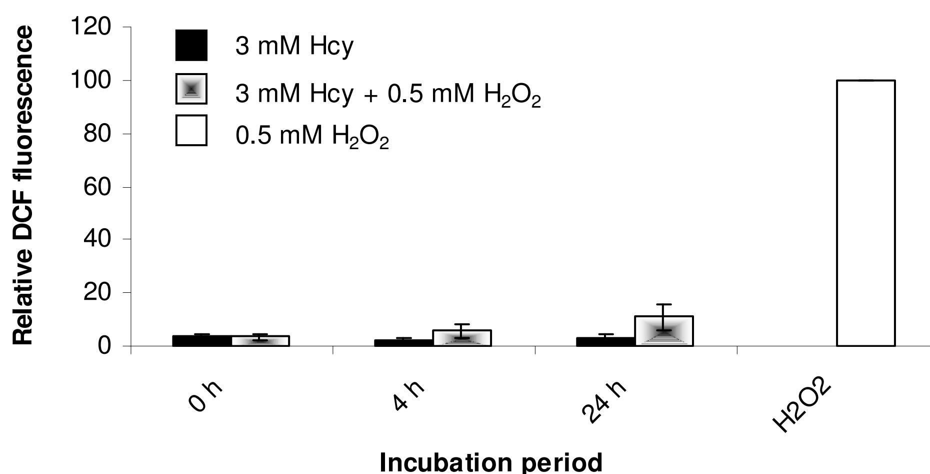


Fig. D-37 Oxidative stress level measured by relative DCF fluorescence after 4 to 24 h incubation of HL60 cells with 3 mM homocysteine (black bars) or pre-treated with homocysteine and challenged with H_2O_2 (grey bars). Results are shown as mean ± S.D. of three independent experiments.

6.1.4 GSH

One possible explanation for the anti-oxidative effect of Hcy is the conversion of Hcy to the cellular antioxidant GSH. Therefore the GSH levels of HL60 and L5178Y cells were analysed by GSH/GSSG assay.

After exposure to 3 mM Hcy, the GSH content of L5178Y and HL60 cells increased within 30 min (Fig. D-38, Fig. D-39). The GSH content of L5178Y cells doubled within 18 h and decreased later on. The GSH content of HL60 cells reached its peak already after 2 h and dropped back to control values within the next 22 h.

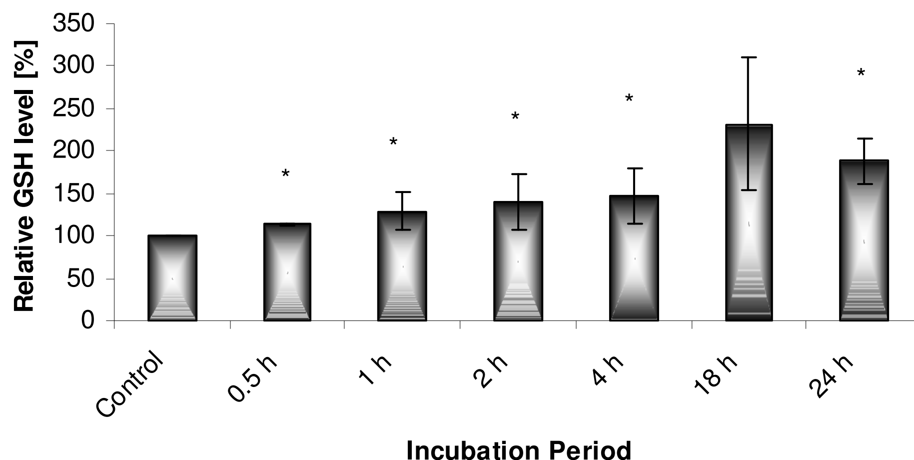


Fig. D-38 Relative amount of GSH in L5178Y cells after incubation with 3 mM homocysteine for 0.5 to 24 h. Results are shown as mean ± S.D. of three independent experiments * $p \leq 0.05$; ** $p \leq 0.01$, *** $p \leq 0.001$

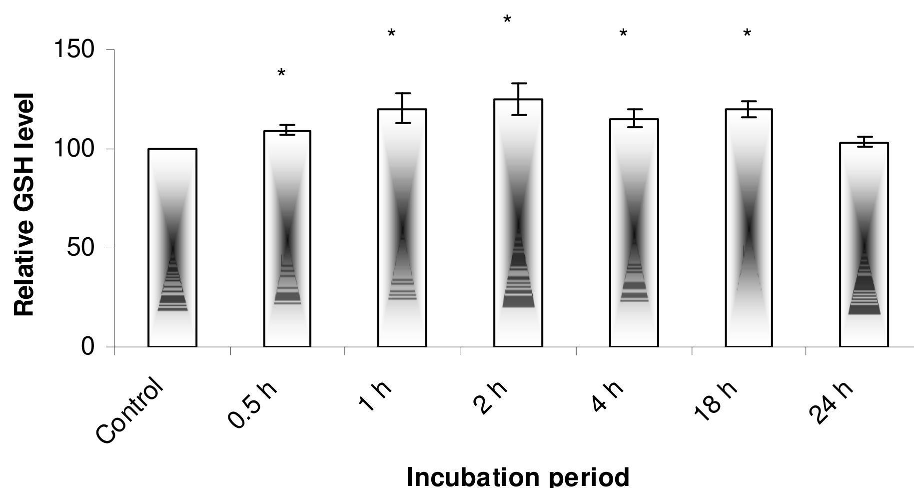


Fig. D-39 Relative amount of GSH in HL60 cells after incubation with 3 mM homocysteine for 0.5 to 24 h. Results are shown as mean ± S.D. of three independent experiments * $p \leq 0.05$; ** $p \leq 0.01$, *** $p \leq 0.001$

6.1.5 Methylation

Another potential mechanism for MN induction is the change of overall DNA methylation. Therefore DNA methylation of L5178Y cells was analysed by flow cytometry and HPLC-MS/MS after incubation with Hcy. No significant changes could be detected by either method, while the positive control 5-azacytidine reduced overall DNA methylation dramatically (Fig. D-40). Additionally, the influence of Hcy-T on overall DNA methylation was determined by HPLC-MS/MS. No significant effect could be observed (Fig. D-41).

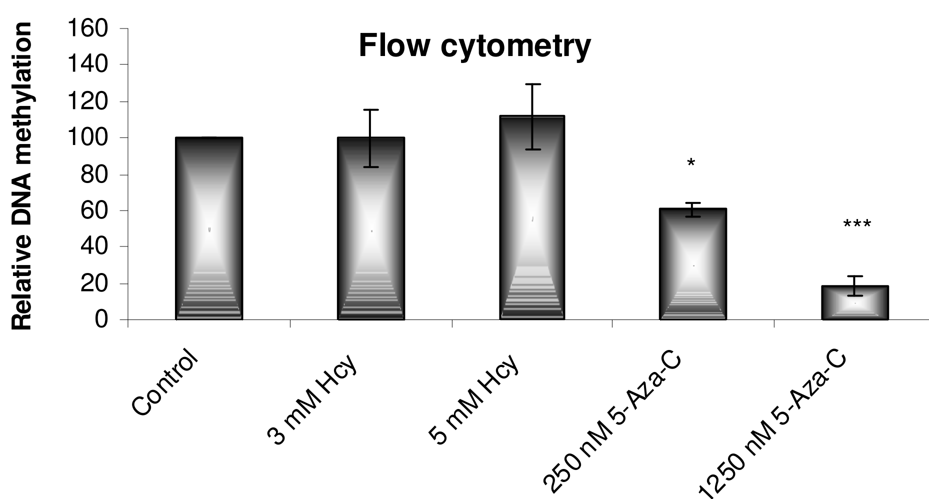


Fig. D-40 Flow cytometric analysis of DNA-cytosine methylation in L5178Y cells after 72 h exposition of homocysteine. The results are shown as relative DNA-cytosine-methylation compared to untreated cells. 5-Azacytidine (5- Aza-C) served as positive control. Results are shown as mean ± S.D. of three independent experiments * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$

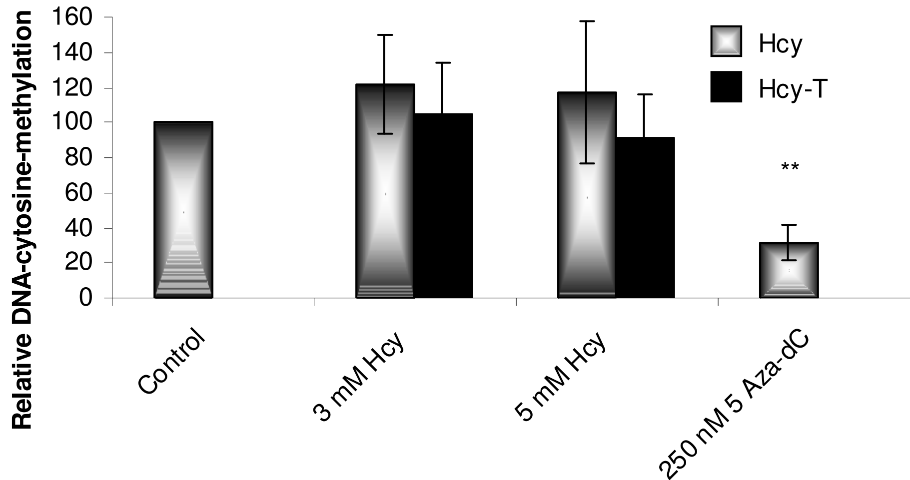


Fig. D-41 Analysis of DNA-cytosine methylation in L5178Y cells after 72 h exposition of homocysteine by HPLC-MS/MS. The results are shown as relative DNA-cytosine-methylation compared to untreated cells. 5-Azacytidine (5-Aza-C) served as positive control. Results are shown as mean ± S.D. of three independent experiments * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$

6.1.6 BrdU

Microscopic analysis of Hcy-treated cells revealed a reduced percentage of mitotic cells. The kind of mitotic interference was specified by BrdU incorporation assay. A 12 h incubation of L5178Y cells with up to 5 mM Hcy did not alter the percentage of cells in specific phases significantly (Fig. D-42). However, after 24 h incubation with 5 mM Hcy the percentage of cells in the G1-Phase was reduced significantly, while the amount of cells in the S-Phase increased (Fig. D-43). Incubation with 3 mM Hcy had the same effect, but did not reach significance.

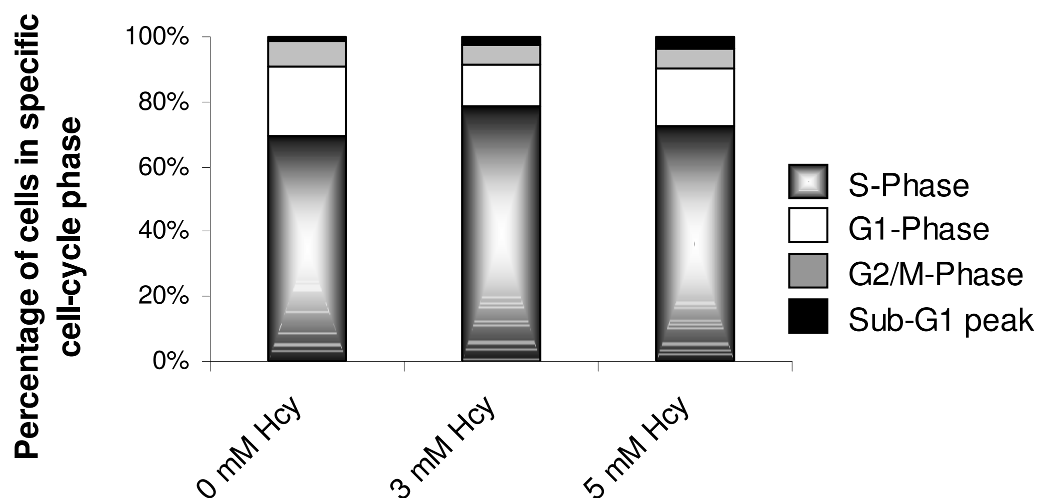


Fig. D-42 Cell cycle analysis of L5178Y cells by BrdU incorporation assay after 12 h exposure to homocysteine. The results are given as percentage of cells in the S-Phase, G1-phase, G2-M phase and sub-G1 peak. Results are shown as mean ± S.D. of three independent experiments * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$

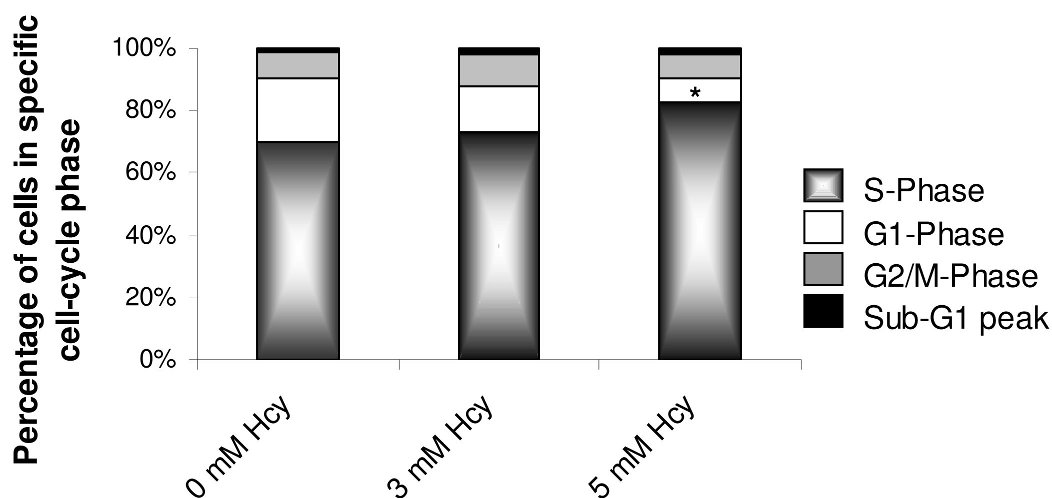


Fig. D-43 Cell cycle analysis of L5178Y cells by BrdU incorporation assay after 24 h exposure to homocysteine. The results are given as percentage of cells in the S-Phase, G1-phase, G2-M phase and sub-G1 peak. Results are shown as mean ± S.D. of three independent experiments * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$

6.2 *Leptin*

6.2.1 Cytotoxicity Testing

L5178Y cells were incubated with 0.1 to 10 µg/ml leptin. Those concentrations had no effect on the cell proliferation, mitotic frequency (data not shown) or apoptosis analysed by flow cytometry (Fig. D-44)

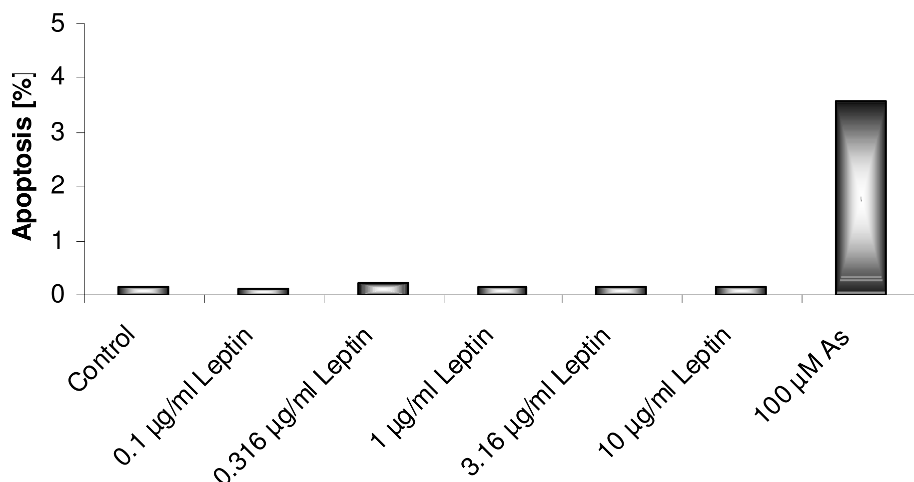


Fig. D-44 Percentage of apoptotic L5178Y cells after 24 h incubation 0.1 – 10 µg/ml $NaAsO_2$.

6.2.2 Genotoxicity Testing

The genotoxic effect of leptin was analysed by MN frequency test (Fig. D-45) and by comet assay (Fig. D-46). Leptin did not influence the MN frequency up to concentration of 10 µg /ml. However, leptin induced DNA damage by comet assay at concentrations as low as 1 µg/ml. As it was not analysed which kind of Ob receptors are presented on the cell surface of L5178Y cells, it could not be elucidated whether the DNA damage was mediated by receptor, or whether leptin was ingested by the cell and interacted directly with DNA.

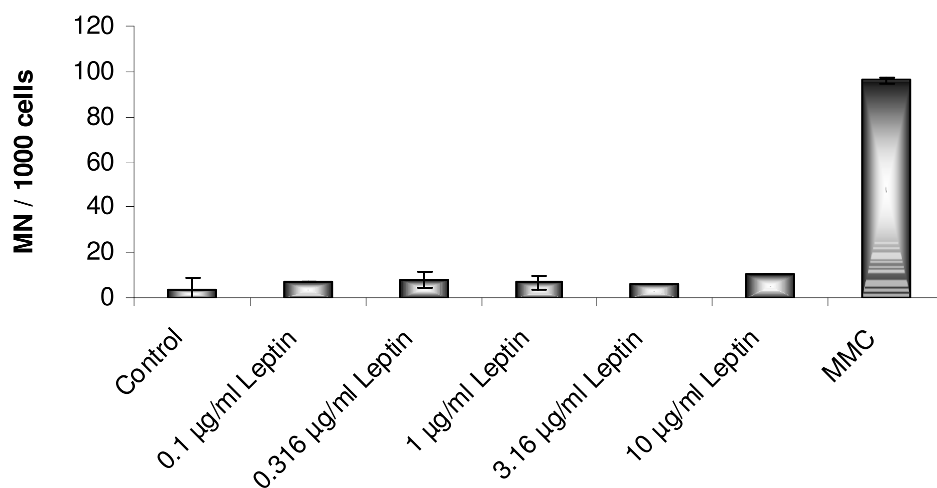


Fig. D-45 Micronucleus induction in L5178Y cells after incubation with leptin for 24 h. Mitomycin C (MMC; 125 ng/ml) served as positive control. Results are shown as mean values between two independent experiments.

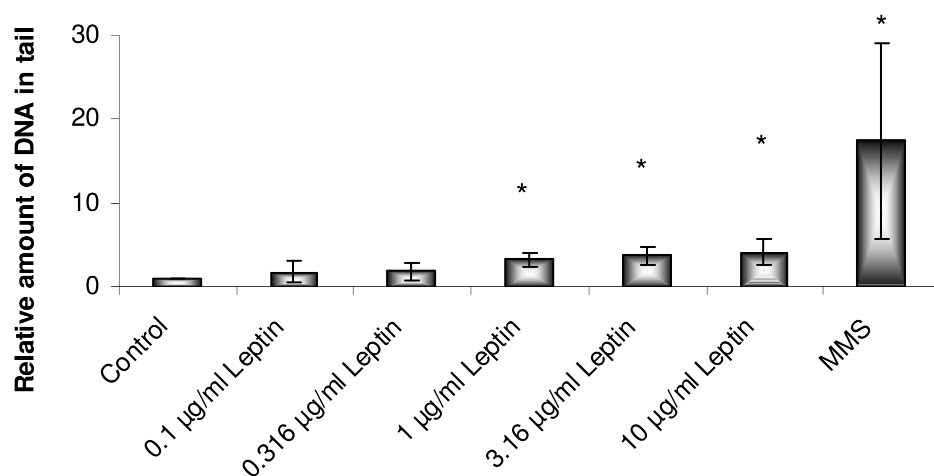


Fig. D-46 DNA damage analysed by comet assay (relative percentage of DNA in tail) after incubation of L5178Y cells with leptin for 24 h. Methyl-methane-sulfonate (MMS; 50 µM) served as positive control. Results are shown as mean of three independent experiments ± standard deviation * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$

As the effect of leptin could only be detected in the comet assay and what is more, as this effect was observed only at concentrations 1000 times higher than the ones in patients, further experiments were abandoned.

6.3 Advanced Glycation End Products

Finally, the genotoxic capacity of AGEs was examined. This was mainly done on porcine proximal tubule cells LLC-PK1. Incubation with freshly synthesized AGEs (CML and MGO) did not influence cell proliferation or number of apoptotic cells, but it induced genomic damage detectable by comet-assay (Fig. D-47). However, the AGEs synthesized by ourselves lost their activity already after one or two weeks, even if stored at -80°C. There were also huge differences between AGEs produced from different BSA batches. These problems prevented the averaging of three consecutive experiments.

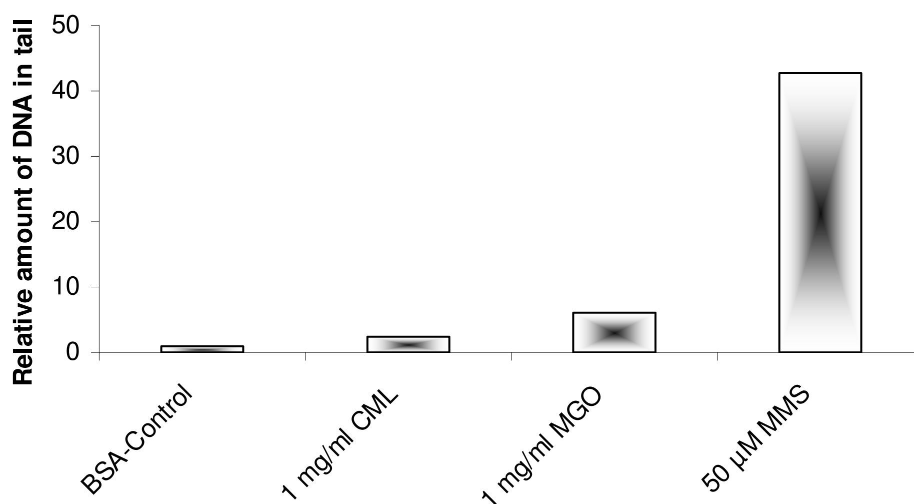


Fig. D-47 Exemplary comet assay of LLC-PK1 cells incubated with AGEs for 24 h.

6.4 Summary of Toxic Effects of Uremic Toxins

A summary of toxic effects of uremic toxins analysed in this study is given in Tab. D-6. Additional information on three further uremic toxins - indole-3-acetic acid, indoxyl sulfate and methylguanidine - is presented in this table. This information was gathered during the master thesis of co-worker Birgit Werner (Werner 2005).

	Hcy	Leptin	AGEs	Indole-3-acetic acid	Indoxyl sulfate	Methylguanidine
Cytotoxicity	+	-	-	+	+	+
Mitotic frequency	+	-	n.d.	n.d.	n.d.	n.d.
Apoptosis	-	-	-	n.d.	n.d.	n.d.
Micronuclei	+	-	n.d.	-	(+)	+
Comet-Assay	-	+	+	+	-	-

Tab. D-6 Summary of cytotoxic/genotoxic effects of uremic toxins on L5178Y or LLC-PK1 cells; +: effect; -: no effect; n.d.: not determined.

7 Effects of Patient Serum

Several uremic toxins exhibited genotoxic features but none of them at concentrations or to an extent which could by itself explain the increased genomic damage in HD patients. Therefore, we analysed whether the increased genomic damage could directly be related to the sum of the known and unknown substances present in the patient serum. Serum samples of HD patients who displayed an increased MN frequency (as analysed by another study (Treutlein, to be submitted), were prepared. Additionally a 10 kDa filtrate of the serum was obtained. This filtrate corresponds to the filtrate of average dialysers. Thereafter, L5178Y cells were incubated with serum or 10 kDa filtrate for 24 h.

In order to mimic the real life situation the patient serum in the media should be as highly concentrated as possible. To evaluate the maximum amount of serum which could be added to L5178Y cells in general without influencing them, preliminary experiments with horse serum, were performed. Addition of up to 30% horse serum to the cell culture media did not alter cell proliferation or appearance.

Unfortunately, L5178Y cells tolerated human serum not in the same way. The addition of 20% patient serum to the normal cell media (containing 10% horse serum) killed all of the L5178Y cells. Heat inactivation of the complement system did not reduce the cytotoxicity.

L5178Y cell resisted to the addition of up to 2% patient serum without showing severe cytotoxic effect (except for the serum of patient one). However, with 3 out of 4 serum samples there was also no increase of MN frequency, even if incubation was maintained for up to one week (Fig. D-48 and Fig. D-49). However, whether this was

due to the strong dilution or the lack of genotoxic capacity could not be elucidated. Solely the plasma of patient one doubled the MN frequency, but it also caused cytotoxicity, the genotoxic effect may be secondary.

Additionally, cells were incubated with 10kDa filtrate. Concentration up to 20% filtrate hardly influenced the cell proliferation or MN frequency (Fig. D-48 and Fig. D-49).

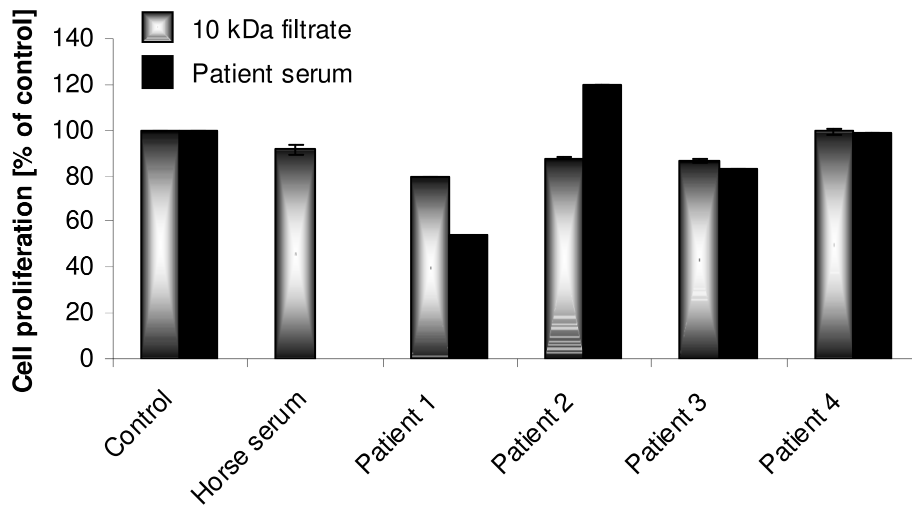


Fig. D-48 Proliferation after incubation of L5178Y cells after incubation of L5178Y cells with 2% patient serum for 1 week (black bars) or with 20% 10 kDa filtrate of the patient serum for 24 h (grey bars).

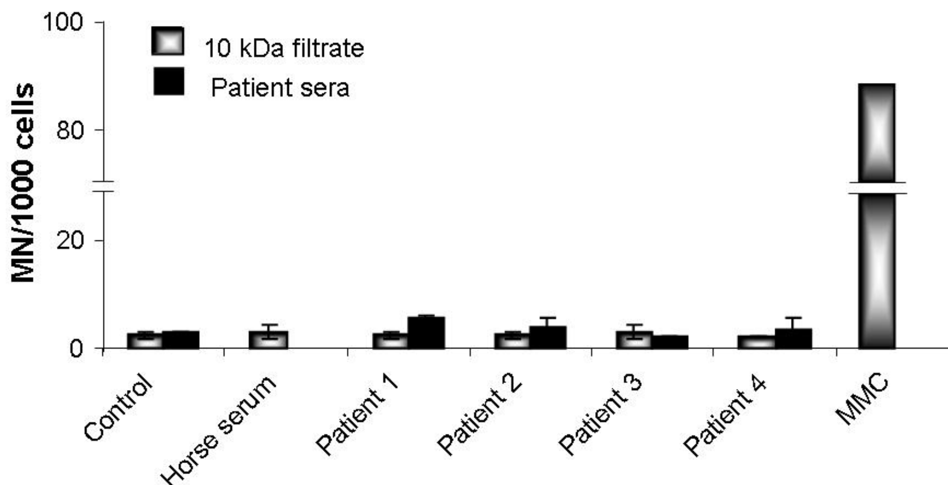


Fig. D-49 Micronucleus induction in L5178Y cells after incubation of L5178Y cells with 2% patient serum for 1 week (black bars) or with 20% 10 kDa filtrate of the patient serum for 24 h (grey bars).

E Discussion

Dialysis patients suffer from increased cancer incidence and increased genomic damage (Maisonneuve, Agodoa et al. 1999; Stopper, Meysen et al. 1999; Stopper, Boullay et al. 2001; Teschner, Garte et al. 2002; Stewart, Buccianti et al. 2003; Vajdic, McDonald et al. 2006). Many causes have been discussed in the literature. However, this study focused on only two:

1. The potentially genotoxic effect of substances leaching from dialysers, and
2. the possible genotoxic effect of selected uremic toxins

1 Dialysers

Optical evaluation of freeze-dried eluates proved clearly that substances are leaching from blood circuits containing dialysers and tubing. HPLC analysis of eluates confirmed this observation. A comparison between the full ion spectra of eluates and pure eluent (ddH_2O or 17.2% EtOH) showed significant changes which could not be attributed to one single substance. Therefore we concluded that several different substances leach from dialysers. It was most likely that these substances consist of mechanical abrasion of the tubing by the flexible-tube pump, production residue like pore filler material glycerol as well as BPA from dialysers or platicizers like DEHP from the tubing.

This is in line with previous studies which report DEHP leaching from PVC blood-tubing (Flaminio, Bergia et al. 1988; Faouzi, Dine et al. 1999; Dine, Luyckx et al. 2000) or BPA leaching from synthetic dialyser membranes (Haishima, Hayashi et al. 2001; Yamasaki, Nagake et al. 2001; Murakami, Ohashi et al. 2007).

1.1 BPA

BPA was detected in all eluates. The extrapolated amounts of leaching BPA ranged from 6.4 ng/ dialyser to 4.3 µg/ dialyser. Several factors contributed to this broad range of leaching BPA: (1.) the type of dialyser, (2.) the batch, (3.) the choice of eluent and (4.) the time period for which the extraction was performed.

As expected a prolongation of the extraction time resulted in an increase of BPA leaching from the blood circuit. This increase was between 10 - 50% of the overall amount except for 2 cases (FX80, Lot-No NCX01130, ddH_2O and 170H, Lot-No S-4402-H-01, 17.2% EtOH) in which the amount stayed nearly the same.

The choice of eluent had a much greater impact on the amount of leaching BPA. The BPA concentration of 17.2% EtOH eluates was up to 47 times higher than the one in comparable ddH_2O eluates. This was a reasonable finding because the extraction capacity of a water/ethanol mixture is much higher in regard to BPA.

The third factor that influenced the amount of leaching BPA - the type of dialyser - was also anticipated. In case of dialysers which consisted of the same material for housing and membranes but differed in the surface area (FX60 vs FX80 and F60S vs HF80S), the amount of extracted BPA was higher in the dialysers with the larger surface area.

More surprising were the huge differences of leaching BPA between single batches of FX80 and FX60 dialysers. Errors of eluate handling, e.g. by insufficiently cleaned glassware, can be ruled out because eluates of the same batch were produced on different days using new equipment. It can therefore be assumed that quality differences during the manufacturing process and differences in the originating polysulfone-granules before extrusion are the underlying cause. In addition the variations of estimated BPA content leaching from dialysers of those batches was more than 40%, while it was generally below 20% in batches from which low amount of BPA were leaching.

Given 17.2% EtOH has similar extraction properties towards BPA as bovine serum - and hence to human serum – those eluates have been used to estimate human exposure. Assuming the worst case an absolute amount of 4.3 µg BPA would be leaching into the blood of the dialysis patient per dialysis session (FX80 dialyser, Lot No: NCX01130, 24 h elution, 17.2% EtOH). If one bases the estimation of additional body burden on an average adult person (5 l blood volume, 70 kg body weight) this leads to an additional 0.86 ng BPA/ml blood or 61.4 ng BPA/kg body weight respectively. Presumably, this calculation overestimates the amount of BPA because a dialysis session lasts only 4 h, not 24 h. This reduction leads to a 21% decrease of leaching BPA in this type of dialyser (Table D-3, page 81).

Some previous studies already evaluated the amount of BPA released from hemodialysers. Haishima et al. analysed four different dialyser types composed of a polycarbonate or polystyrene housing in combination with cellulose acetate or polysulfone hollow fibres (Haishima, Hayashi et al. 2001). They detected 3.78 to 141.8 ng BPA/module when using ddH_2O as a solvent, 140.1 to 2,090 ng BPA/module when using bovine serum as solvent or 153.3 to 2,090 ng BPA/module

when using 17.2 EtOH as solvent. The values obtained with ddH_2O correspond to our findings (6.4 to 71.3 ng BPA/ module). The amounts of BPA extracted by 17.2% ethanol are also within the range reported by Haishima et al. for all but a single batch of FX80 dialysers (Lot No: NCX01130). This batch released roughly twice as much BPA than the highest report by Haishima. As Haishima et al. performed the extraction at room temperature and not - like we did - at 37℃ - 38℃ (the temperature normally used during dialysis), washed the hemodialysers 3 times prior to BPA extraction, used a slower flow rate (19 ml/min instead of 230 ml/min) and a shorter extraction period (16 h instead of 24 h), this slight discrepancy is easily explained.

Yet another group detected a maximum of 1.14 ng BPA/ dialyser or nothing in the effluent of 5 different dialysers (Yamasaki, Nagake et al. 2001). However, they neither circulated the water, nor did they specify how long it stayed inside the dialyser or which temperature was used. They also failed to detect BPA in whole blood samples of one HD group. This is in contrast to the various studies which report BPA even in blood samples of the normal population (see below). Furthermore no information on the HPLC method used is given; therefore it is not possible to compare it to our results.

A recent study reports rather high concentrations of BPA (83.3 ng/10 mg hollow fibres) released by polysulfone or PEPA polyester-polymeralloy (Nikkiso, Japan) (122.5 ng/10 mg hollow fibre) hollow fibres (Murakami, Ohashi et al. 2007). However, the authors obtained these results by crushing the hollow fibres and dissolving them in DMSO. Therefore these results are not comparable to the *in vivo* situation or our data.

The same study also analysed the amount of BPA leaching from dialysers with polysulfone membranes directly into the blood of 15 HD patients. The BPA level of those patients increased from 4.83 ± 1.94 ng/ml blood prior to dialysis to 6.62 ± 3.09 ng/ml thereafter (Murakami, Ohashi et al. 2007). This equates to an additional BPA body burden of 1.79 ng/ml blood which is roughly twice as much as our worst case estimation. However, another experiment with the same patients and the same dialysers resulted in an increase of only 0.18 ng BPA/ml blood during dialysis. This corresponds to 20 % of the increase estimated by our worst case scenario. Unfortunately, the paper does not explain the discrepancy between their measurements. It can therefore be assumed that the amount of BPA released was

midway between 0.18 and 1.8 ng/ml blood, which is also what our experiments predicted.

In order to evaluate the risk which BPA poses to HD patients, the amount has to be compared to the BPA burden of average humans. Over the last few years more than a dozen studies have measured BPA in blood of men and women from several countries at different ages. The analysis included a variety of analytical methods like ELISA, LC-MS, GC-MS or HPLC. Depending on the method, the BPA content ranged from 0.3 – 20 ng/ml blood (Inoue, Kato et al. 2000, Ikezuki, 2002; Schönfelder, Wittloht et al. 2002; Takeuchi and Tsutsumi 2002; Sugiura-Ogasawara, Ozaki et al. 2005; for review Vandenberg, 2007). Also, BPA has no tendency to accumulate; the half life in the body is estimated to be less than one day (Pottenger, Domoradzki et al. 2000; Takahashi and Oishi 2000). Therefore the contribution of HD to the overall body burden is only marginal and the risk for the patient practically non-existent.

This is especially true if one compares the estimated additional body burden of 61.4 ng/kg bw to the tolerable daily intake of 50 µg/kg bw established by the European Food Safety Agency (EFSA 2006). Of course it has to be considered that the TDI was only established for oral exposure and not intravenous exposure. After oral uptake, BPA is rapidly transformed to BPA glucoronide during the first pass metabolism in the gut wall and the liver. The glucoronide form lacks endocrine activity. If BPA leaches directly into the blood this metabolic biotransformation takes longer and more BPA is available as parent BPA. However, bioavailability and activity of parent BPA is reduced because it binds rapidly to human plasma proteins.

Even if this was not the case, there is still a safety factor of nearly 1000 between the oral TDI and the actual *intravenous* exposure, which can be regarded as sufficient to minimise the potential risk.

1.2 DEHP

Although DEHP could be detected in the eluates, the amounts were too low reach the limit of quantification. This was surprising as we expected quantifiable amounts of DEHP to leach from the blood tubing system. This expectation originated from studies which report that patients undergoing maintenance hemodialysis retain 3.6 - 59.6 mg DEHP per dialysis session (average 16.4) (Faouzi, Dine et al. 1999; Dine, Luyckx et al. 2000). An older study found even higher concentrations 23.8 - 360 mg leaching from the dialyser into the blood during a single session (average

105 mg) (Pollack, Buchanan et al. 1985). Given that leaching plasticizers – especially leaching DEHP – have been recognized as a possible risk to human health, there has been some effort to reduce the amount of leaching DEHP. Therefore values of the more recent study will probably reflect the present situation more accurately. Assuming the average amount of leaching DEHP would had been 16 mg, the concentration in the 4 ml concentrated eluate would have been 4 mg/ml which is considerably higher than the limit of quantification (ca. 0.07 µg/ml).

In order to explain this discrepancy, it has to be considered that the extraction conditions of the present study differ from *in vivo* ones. In previous studies, the DEHP was measured directly in the blood of HD patients. The complete amount of leaching DEHP is stated as the difference between DEHP prior to a dialysis session and thereafter. We on the other hand used ddH_2O and 17.2 % EtOH for DEHP extraction. As the extraction properties of blood and water are very different, it is possible that our dialysis modalities failed to extract any DEHP. This seems plausible - at least in regard to the ddH_2O eluents - because DEHP solubility in water is very poor (41 µg/l at 25°C (Leyder and Boulanger 1983)). However, this does not explain the complete absence of DEHP in the ethanol eluates because DEHP migrates from PVC/DEHP blends into ethanol/water mixtures (Kim, Kim et al. 2003). Apart from the different eluent we also used only 1.1 m of tubing instead of several meters in the real HD, which did lead to reduced amounts of potentially leaching DEHP. Finally, our analytical method required the solution of DEHP in organic solvents, in our case acetonitrile. During this process the eluents were diluted 1:10. All these factors may have contributed to lower the concentration of DEHP. However, it can be concluded that less DEHP was leaching from the tubing system used in this study than from tubing systems of previous reports.

2 Toxicity of Eluates

Even though BPA was detected in each eluate, extensive toxicity testing yielded no evidence for cyto- or genotoxicity. This is reassuring because the eluate concentration reached in cell culture media was 25 times (EtOH eluates) to 50 times (ddH_2O eluates) higher than the expected concentration in the blood of HD patients. This means there is an additional margin of safety.

Especially comforting is the lack of disturbed mitosis or reduced mitotic frequency as well as the lack of MN in L5178Y cells exposed to eluates, given that BPA is

known to be a meiotic aneugen in female mice (Hunt, Koehler et al. 2003; Susiarjo, Hassold et al. 2007). Other toxic effects of BPA include aneuploidy, disturbances in the microtubule assembly, MN formation and DNA damage detectable by comet assay *in vitro*, albeit at > 100 µg/ml while the BPA concentration reached in our test system did not exceed the low ng range (Pfeiffer, Rosenberg et al. 1997; Tsutsui, Tamura et al. 1998; Lee, Kwon et al. 2003). Therefore an induction of genomic damage due to substances leaching from blood circuits ca not be expected.

However, EtOH eluates induced a slight increase in cell proliferation of the estrogen sensitive cell line MCF-7. This increase of cell proliferation cannot solely be attributed to BPA. A comparison to growth induction of pure BPA showed that 7.2 ng/ml BPA are necessary to increase the proliferation of MCF-7 cells significantly, while the highest BPA concentration due to eluates was 5.8 ng/ml media (FX80). Even EtOH eluates containing only a small amount of BPA (0.03 ng/ml media) increased the cell proliferation. It can therefore be assumed that substances other than BPA exhibited this estrogenic activity, or that BPA and another substance acted synergistically.

We did not analyse for further substances but other groups report that repeated flexion and compression of the tubing segment of HD by rollers of the peristaltic pumps leads to abrasion of particles into the extracorporal circuit (Barron, Harbottle et al. 1986).

Regardless of which substance of the eluate exhibits this estrogenic activity, the relevance for the enhanced cancer incidence is limited. If the increased cancer incidence was induced by estrogenic activity, one would assume that cancers of estrogen responsive tissue (e.g. breast cancer) were the most prominent. However, this is not the case in epidemiological studies; the incidence of breast cancer incidences does not differ from the general population in a statistically significant way (Maisonneuve, Agodoa et al. 1999; Teschner, Garte et al. 2002; Stewart, Buccianti et al. 2003).

3 Uremic Toxins

3.1 Homocysteine

Low millimolar levels of Hcy induced MN in several cell lines. Similarly, preliminary *in vitro* studies on pooled human lymphocytes showed an increased MN

frequency after incubation with Hcy (Crott and Fenech 2001). This is in line with *in vivo* observations which correlate increased levels of Hcy in serum with an increase in MN frequency (Fenech, Dreosti et al. 1997; Fenech, Aitken et al. 1998; Fenech 1999). So far the mechanism by which Hcy induces MN is not known. One of our hypotheses was that MN induction is connected to disturbances of the cell cycle. Disturbance of the cell cycle progression, i.e. an increased percentage of cells in the S-phase were observed at the same concentration as MN. A prolonged S-phase can result from changes in the level of DNA-cytosine-methylation. This is interesting in the present context, since the DNA of dialysis patients, suffering from homocysteinemia is often hypomethylated (Ingrosso, Cimmino et al. 2003) or shows aberrant methylation patterns (Zaina, Lindholm et al. 2005). Increased plasma levels have also been associated with increased DNA methylation of lymphocytes in healthy subjects (Yi, Melnyk et al. 2000; James, Melnyk et al. 2002). The hypothesis for the mechanism leading to hypomethylation is as follows:

The conversion of SAH to Hcy is a readily reversible reaction, which strongly favours SAH synthesis instead of hydrolysis. The reason for the normal hydrolysis reaction is the fast product removal (Yi, Melnyk et al. 2000). If the Hcy concentration increases, the SAH concentration increases as well (Hoffman, Marion et al. 1980). SAH in turn is a potent inhibitor of the SAM methyltransferase (Hoffman, Marion et al. 1980). If SAM-methyltransferases are inhibited, the methyltransferation of SAM to DNA and other methyl acceptors drops. This leads to DNA hypomethylation.

However, neither Hcy nor Hcy-T treatment up to cytotoxic concentrations caused an overall DNA-cytosine-methylation change *in vitro*. This is in contrast to the situation in ESRD patients, in which hyperhomocystemia is correlated with hypomethylation (Ingrosso, Cimmino et al. 2003). One possible explanation could be a fast removal of Hcy by the transsulfuration pathway in the cell lines used within this study. This would prevent the accumulation of SAH, the inhibitor of the SAM methyltransferase.

Another possible mechanism for genotoxicity is the generation of ROS. Indeed, it has been proposed that some of the adverse effects associated with hyperhomocysteinemia may be due to the generation of ROS (Au-Yeung, Woo et al. 2004; Perez-de-Arce, Foncea et al. 2005). However, prior treatment of L5178Y cells or co-incubation with oxygen scavengers like NAC did not reduce the MN frequency. The comet assays of Hcy-treated cells yielded no evidence for oxidative DNA

damage. Hcy treatment had also no impact on the amount of ROS in HL60 cells, as detectable by DCFH-DA analysis. This is in line with prior observations in HUVECS (Outinen, Sood et al. 1998) and LLC-PK1 cells (Nakanishi, Akabane et al. 2005), in which Hcy failed to elicit an oxidative stress response. Thus the generation of oxidative stress by Hcy may be cell-type specific and /or dependent on experimental conditions. This idea is supported by publications stating that Hcy (50 µM) increases the ROS production of bovine aortic endothelial cells (BAEC) under high glucose but not under normal growth condition (Sethi, Lees et al. 2006). It can inhibit as well as promote LDL oxidation depending on the experimental conditions (Lynch, Campione et al. 2000).

Interestingly, pre-treatment with Hcy caused even a considerable reduction of oxidative stress in cells challenged by H_2O_2. This effect is probably due to a conversion of Hcy to the major redox buffer of cells: GSH. This conversion takes place in the transsulfuration reaction which provides the direct link between Hcy and GSH (Mosharov, Cranford et al. 2000). In case of oxidative stress, the Hcy flux through the transsulfuration pathway in liver cells is 2-3 times enhanced (Mosharov, Cranford et al. 2000). This precedence over the transmethylation pathway is controlled by the methionine synthase, whose activity is decreased under oxidizing conditions (Chen, Pettersson et al. 1998), and the cystationine β-synthase, whose activity increases under oxidizing conditions (Taoka, Ohja et al. 1998).

In fact, the GSH levels of HL60 and L5178Y cells started to increase as early as 30 min after incubation with Hcy. A similar increase of total intracellular GSH could be observed in BAEC after incubation with 5 mM Hcy (Upchurch, Welch et al. 1997) or 50 µM Hcy (Sethi, Lees et al. 2006) or in DAMI cells (human megakaryocytic cells) after incubation with 1 mM – 10 mM Hcy (Austin, Sood et al. 1998). However, Upchurch et al. report that this was accompanied by a decreased glutathione peroxidase activity.

After these experiments, ROS production or disturbances of the DNA methylation can be ruled out as mechanisms for MN induction in L5178Y or HL60 cells. One remaining explanation may be the prolonged S-phase after incubation with Hcy. This may possibly lead to disturbances of the mitosis, which may lead to MN formation. However, MN induction in L5178Y cells started after addition of 3 mM Hcy, while the first significant cytotoxicity was observed at 4 mM Hcy. This slight difference could be explained by the possibility that Hcy does not act directly genotoxic but non genotoxic

mechanisms are involved. The increased MN frequency of HL60 cells cannot be explained in this way because no cytotoxicity was observed in this concentration range.

3.1.1 Consequences for the Patient

Hcy exhibits genotoxic or antioxidant effects at concentrations significantly higher than the levels observed in the serum of ESRD patients (up to 220 µM; (Perna, Satta et al. 2006)). Although this may lead to the conclusion that these effects are negligible for the patients, one can not rule out that – upon local accumulation in certain tissues or cell types – toxicologically relevant levels may be reached. Additionally, other more sensitive endpoints - e.g. gene expression measurements – might detect Hcy-induced alterations at lower doses.

One particular interesting hypothesis can be put forward in regard to the conversion of Hcy to GSH. In HD patients additionally suffering from chronic malnutrition inflammation complex a high rather than a low Hcy level is correlated to longer survival (Kalantar-Zadeh, Block et al. 2004; Wrone, Hornberger et al. 2004; Ducloux, Klein et al. 2006). In those patients, the GSH level drops due to a disturbed thiol homeostasis (Wlodek, Smolenski et al. 2006). Considering this, a conversion of Hcy to GSH could enhance the oxidant defence, which contributes to the better survival. The same mechanism has been observed in malnourished children – their chances for survival are better when given GSH (Becker, Pons-Kuhnemann et al. 2005).

3.2 Homocysteine-Thiolactone

The cytotoxic and genotoxic effects of the Hcy derivate Hcy-T were also analysed. Hcy-T was more cytotoxic towards L5178Y cells than Hcy. A decline of cell proliferation could be observed at 1 mM Hcy-T. The MN frequency increased at the same time. This is in line with studies of HL60 cells, in which severe cell death occurred at concentrations of 500 – 1000 µM (Huang, Huang et al. 2002). Our experiments with Hcy-T were troubled by the short half-life of 1 h and the purchasable form which was only as hydrochloride. This resulted in an acidification of the cell culture media which is problematic. Furthermore, the concentrations of Hcy-T in plasma are even 3 powers lower than the ones of total Hcy (Chwatko and Jakubowski 2005) its relevance for the *in vivo* situation is therefore highly unlikely.

3.3 Advanced Glycation End-Products

The two AGEs tested (MGO-BSA and CML-BSA) induced some genotoxicity detectable by comet assay. However, the degree of genomic damage varied highly between the batches of synthesized AGEs. Additionally, newly synthesized AGEs lost their genotoxic capacity within days even if stored at -80℃. Some of these problems have been overcome in another study and the genotoxic activity of AGEs on LLC-PK1 and additional cell lines has been confirmed (Schupp, Schinzel et al. 2005). However, the concentrations reached in cell culture are significantly higher (1 mg/ml versus 110 ng/ml) than in the HD patients. Nevertheless, AGEs are a heterogeneous group of proteins; therefore it is possible that other AGEs are even more genotoxic than the ones tested. The mechanism of genotoxicity was not analysed in this study, however it is hypothesised that AGEs act *via* the RAGE receptor. Activation of RAGE leads to activation of NF-κB, which leads to increased cytokine, chemokine growth factor and ROS production (Sebekova, Wagner et al. 2007). Finally, this leads to oxidative DNA damage.

3.4 Leptin

Leptin did not induce any cytotoxicity at concentrations of up to 10 µg/ml, while it did induce some genomic damage detectable by comet assay starting at 1 µg/ml. This is roughly 10 times as much as the average leptin concentration in HD patients (Vanholder, De Smet et al. 2003). The maximum amount of leptin detected in uremic patients is 0.49 µg/l (Vanholder, De Smet et al. 2003). Therefore it is possible that leptin contributes to the genomic damage observed in HD patients. This is supported by the observation of leptin levels correlating with the peripheral genomic damage of HD patients (Horoz, Bolukbas et al. 2006).

3.5 Serum of Dialysis Patients

None of the uremic toxins tested within this study provides a sufficient explanation for the increased genomic damage observed in ESRD patients.

In order to evaluate whether uremic toxins are responsible for this problem or play a minor part, L5178Y cells were incubated with serum or 10 kDa filtrate of HD patients with increased MN frequency. Addition of serum or 10 kDa filtrate did not induce cyto- or genotoxicity up to a concentration of 2% or 20%, respectively. However, higher concentrations were severely cytotoxic. This severe cytotoxicity

cannot be attributed to the uremic toxins alone. It is very likely that this cytotoxicity was due to inadequate inactivation of the complement system.

The non-cytotoxic concentration of 2% serum in media is considerably lower than the concentration to which lymphocytes are exposed. Therefore the results of tests with patient serum or 10 kDa filtrate do not allow any conclusion as to whether substances present in the serum are responsible for the genomic damage observed in dialysis patients.

4 Conclusion

Based on the *in vitro* tests with eluates and the chemical analysis of eluates, it can be concluded that substances leaching from dialysers pose no risk for the HD patients - at least regarding the toxicity endpoints analysed.

It is therefore unlikely that substances leaching from dialysers are responsible for the genomic damage and increased cancer incidence. This holds especially true as most of the increased cancer incidence is observed within the first year after the start of dialysis (Stewart, Buccianti et al. 2003). Cancer is a slowly progressing disease which takes years to decades to develop. The rapid diagnosis after the start of HD, supports the argument for detection because of better surveillance, not for cancer development due to substances leaching from dialysers.

It is therefore more likely that uremic toxins play a role as they start to accumulate as renal filtration decreases. Apart form the uremic toxins discussed above, several additional uremic toxins have been tested for genotoxicity (indole-3-acetic acid, Indoxyl sulphat and methylguanidine). All of them were genotoxic *in vitro*, albeit at (much) higher concentrations than the ones reached in HD patients (Werner, 2005). This means that neither of those uremic toxins is sufficient to explain the increased genomic damage or cancer incidence observed in dialysis patients.

Therefore it is reasonable to assume that the increased cancer incidence of HD patients is a multifactorial problem. Other factors which certainly contribute to the problem are:

1. Increased ROS production due to bio-incompability of dialysis membranes
2. an impaired DNA repair mechanism
3. an impaired antioxidant system
4. an impaired immune system

Still an effect of uremic toxins cannot be dismissed easily. Even though the concentrations in patients are generally lower than the concentrations exhibiting genotoxicity *in vitro*, chronic exposure, the special susceptible of certain organs/cells and synergistic effects of the various uremic toxins may lead to genotoxicity at lower concentrations. Uremic toxins may also contribute to the DNA damage indirectly. The accumulation of uremic toxins may impair the immune system, or disturb DNA repair. It should therefore be examined whether patients may profit from an earlier onset of dialysis or whether the disadvantages may outweigh the profit.

Overall, contributions of uremic toxins to the overall genomic damage seem likely, as the reduction of uremic toxins by the more effective daily dialysis (compared to the standard HD three times a week) was found to reduce the genomic damage observed in dialysis patients.

F Zusammenfassung

Patienten, die an terminaler Niereninsuffizienz leiden und mittels Hämodialyse behandelt werden, weisen einen erhöhten Genomschaden auf. Dieser könnte ursächlich für die erhöhte Krebsinzidenz dieser Patientengruppe sein.

Eine der möglichen Ursachen für den erhöhten Genomschaden stellt die Akkumulation genotoxischer Substanzen im Blut der Patienten dar. Diese Substanzen können prinzipiell aus zwei unterschiedlichen Quellen stammen. Erstens besteht die Möglichkeit, dass während der Dialyse Substanzen aus den Dialysatoren, dem Blutschlauchsystem oder gar aus verunreinigtem Dialysat in das Blut der Patienten übertreten. Zweitens führt der Verlust der Nierenfunktion zu einer stark verminderten Exkretion harnpflichtiger Substanzen. Diese Substanzen akkumulieren im Blut und bilden, sofern sie ein toxisches Potential besitzen, die Gruppe der so genannten urämischen Toxine. Einige dieser urämischen Toxine sind potentiell auch genotoxisch.

Im Rahmen der vorliegenden Dissertation wurden exemplarische Vertreter der urämischen Toxine auf ihre genotoxische Wirkung hin untersucht. Außerdem wurde analysiert, ob Substanzen aus Dialysatormembranen oder dem Blutschlauchsystem austreten und in *in vitro*-Toxizitätstests Effekte zeigen. Der Fokus der Analytik lag hierbei auf dem Nachweis von Bisphenol A, dem Hauptbestandteil verschiedener Kunststoffe die für Dialysatoren und Dialysatormembranen verwendet werden, sowie auf Diethylhexylphthalat (DEHP), welches als Weichmacher vielen Blutschlauchsystemen beigesetzt wird.

Hierfür wurde der Dialysevorgang mit Hilfe von 5 verschiedenen Dialysatortypen und PVC-Blutschläuchen imitiert. Als Extraktionsmittelmittel wurde doppelt destilliertes Wasser verwendet bzw. eine 17.2%-ige Ethanol/Wasser-Mischung, die gegenüber BPA ein ähnliches Extraktionsvermögen besitzt wie Blut. Die Temperatur, Dialysedauer und Fließgeschwindigkeit entsprach weitgehend realistischen Dialysemodalitäten. Auf diese Weise gewonnene Eluate wurden mittels HPLC-MS/MS Analysen auf ihren Bisphenol A sowie Diethylhexylphthalat-Gehalt untersucht. Während der DEHP-Gehalt in keinem der Eluate das Quantifizierungslimit überschritt, wurden in allen Eluate zumindest Spuren von BPA nachgewiesen.

Aus diesen Messungen ließ sich abschätzen, dass je nach Dialysedauer, Dialysatortyp und Extraktionsmittel zwischen 6,4 ng und 4,3 µg Bisphenol A pro Dialysator austraten. Die Menge an nachgewiesenem Bisphenol A war dabei in den mit 17.2% Ethanol gewonnenen Eluaten deutlich höher als in den Wasser-Eluaten. Des Weiteren zeigten sich deutliche Unterschiede zwischen den einzelnen Dialysatortypen und Dialysatorchargen. Legt man für die Berechnung der zusätzlichen Bisphenol A-Belastung eines Dialysepatienten den ungünstigsten Fall, d.h. die höchste gemessenen BPA-Konzentration zugrunde, so erhält man einen Wert von 0,84 ng/ml Blut (5 l) oder 61,4 ng/kg Körpergewicht (70 kg Person) pro Dialyse.

Um das daraus resultierende Gefahrenpotential für den Dialysepatienten abzuschätzen, mussten diese Werte mit den bisher in der Normalbevölkerung gemessenen BPA-Plasmaspiegeln verglichen werden. Diese lagen zwischen 0,3 und 18,9 ng/ml Blut. Die zusätzliche Belastung durch die Dialyse liegt also eher im unteren Bereich der Belastung der Normalbevölkerung. Außerdem besitzt Bisphenol A eine kurze Halbwertszeit (> 1 Tag) und weist keine Tendenz zur Bioakkumulation auf. Die zusätzlich Belastung von im schlimmsten Fall 61,4 ng/kg Körpergewicht liegt außerdem noch mehr als den Faktor von 800 unter den derzeitig gültigen Grenzwerten der Europäischen Behörde für Lebensmittelsicherheit (EFSA) für die täglich tolerierbare orale Aufnahme (50 µg/kg Körpergewicht).

Da jedoch weitere - im Rahmen dieser Arbeit nicht näher identifizierte – Substanzen in den Eluaten vorhanden waren, konnte eine Gefährdung der Patienten zunächst nicht gänzlich ausgeschlossen werden. Daher wurden zusätzlich *in vitro* Zyto- und Genotoxizitätstests durchgeführt. Die Konzentration der Eluate im Zellkulturmedium war dabei 25 (Ethanoleluate) bis 50 (Wassereluate) mal höher als die Konzentration, welche im Blut erreicht werden könnten. In keinem dieser Tests ließ sich ein Hinweis auf eine zytotoxische oder genotoxische Wirkung finden. Es konnte lediglich eine leichte östrogene Aktivität der mit Ethanol gewonnenen Eluate nachgewiesen werden. Da BPA tatsächlich östrogene Kapazität besitzt, wurden vergleichenden Versuchen mit reinem BPA durchgeführt. In diesen konnte erst ab 7,2 ng/ml ein signifikanter östrogener Effekt nachgewiesen werden. Da die BPA Konzentration durch Eluate im Medium nur zwischen 0,03 ng/ml und 5,8 ng/ml lag konnte dieser Effekt nicht ausschließlich auf austretendes BPA zurückzuführen sein. Trotz dieser leichten östogenen Aktivität der Eluate kann das Gefahrenpotential für

den Dialysepatienten als minimal eingeschätzt werden. Xenoöstrogene können zwar durchaus bei der Krebsentstehung eine Rolle spielen, allerdings wäre dann eine erhöhte Tumorinzidenz von hormonresponsiven Geweben, wie z.B. Brustgewebe, zu erwarten. Dies ist bei Dialysepatienten jedoch nicht der Fall.

Der zweite Teil der Arbeit fokussierte auf genotoxische Untersuchungen exemplarischer urämischer Toxine. Da im Moment mehr als 90 verschiedene urämische Toxine bekannt sind, musste zunächst eine Auswahl potentiell genotoxischer Kandidaten getroffen werden. Der Schwerpunkt lag dabei auf Homocystein, einem Zwischenprodukt des Methioninkreislaufs. Ein erhöhter Homocysteinspiegel im Plasma von gesunden Menschen korreliert mit einer erhöhten Mikrokernfrequenz. Auch in den im Rahmen dieser Studien durchgeführten *in vitro* Versuchen führte die Exposition verschiedener Zelllinien mit Homocystein zu einer erhöhten Mikrokernfrequenz. Allerdings erhöhte erst eine Inkubation mit 3 mM Homocystein die Mikrokernfrequenz in diversen Zelllinien signifikant, während der Homocysteinspiegel von Dialysepatienten nur in sehr schweren Fällen über 100 µM ansteigt. Im Kometen-Test (einem weitern Genotoxizitätstest) ließ sich jedoch bis zu zytotoxischen Konzentrationen kein erhöhter Genomschadennachweisen.

Mikrokerne entstehen häufig durch Störungen des Spindelapparates. Dies macht sich u. U. in einer Störung des Zellzyklus bemerkbar. Jedoch übte Homocystein erst ab einer Konzentration von 5 mM Homocystein einen statistisch signifikanten Einfluss auf den Zellzyklus aus. Es erhöhte den prozentualen Anteil von Zellen in der S-Phase. Da ein erhöhter Homocysteinspiegel bei Dialysepatienten auch mit einer verminderten DNA-Cytosin-Methylierung korrelierte, wurde überprüft, ob dies für die Verlängerung der S-Phase verantwortlich sein könnte. Konzentrationen bis zu 5 mM änderten die DNA-Cytosin-Methylierung jedoch nicht. Höhere Konzentrationen wurden nicht untersucht, da sie sich als zytotoxisch erwiesen.

Eine weitere Ursache für Genomschaden ist häufig die Entstehung von freien Radikalen in der Zelle. Zugaben des Radikalfängers N-Acetylcystein verminderte jedoch nicht die Mikrokernentstehung. Auch die Messung reaktiver Sauerstoffspezies mittels Durchflusscytometrie erbrachten keinen Hinweis auf oxidativen Stress. Im Gegenteil, durch Wasserstoffperoxyd ausgelöster Radikalstress wurde durch Co-Inkubation mit Homocystein deutlich reduziert. Dies lag vermutlich an der Umwandlung von Homocystein zu dem intrazellulären Antioxidanz Gluthathion.

Radikalstress konnte als Ursache für die Mikrokernentstehung also ausgeschlossen werden.

Des Weiteren wurden Mikrokerntests mit dem Homocystein Derivat Homocystein-Thiolacton durchgeführt. Wenn Homocystein bei der Proteinbiosynthese irrtümlicherweise an Stelle von Methionin aktiviert wird, wandelt es die methionyl-tRNA in Homocystein-Thiolacton um. Daher steigt mit einem erhöhten Homocysteinspiegel auch der Homocysteine-Thiolactonspiegel in der Zelle. Inkubation mit 1 mM Homocystein-Thiolacton erhöhte die Mikrokernfrequenz *in vitro* signifikant. Allerdings zeigte sich gleichzeitig eine deutliche Zytotoxizität, wodurch ein direkter genotoxischer Mechanismus nicht eindeutig nachgewiesen werden konnte.

Weitere interessante urämische Toxine sind die so genannten "Advanced Glycation End-Products" (AGEs). Sie entstehen durch die nicht-enzymatische Reaktion von reduzierenden Zuckern mit freien Aminogruppen von Peptiden oder Proteinen. AGEs sind also eine sehr heterogene Gruppe von Proteinen. Zwei exemplarische Vertreter wurden synthetisiert und im Komten-Test eingesetzt. Dabei zeigte sich eine gewisse Genotoxizität, die allerdings sehr chargenabhängig war.

Als letztes wurde die toxische Wirkung des potentiellen urämischen Toxins Leptin untersucht. Leptin ist ein Hormon, welches hauptsächlich von Adipocyten hergestellt wird und für die Regulation der Nahrungsaufnahme und des Energieverbrauchs zuständig ist. In der Zellkultur induzierte Leptin einen ab einer Konzentration von 1µg/ml Genomschäden, die im Kometen-Test nachweisbar waren. Es induzierte jedoch keine Mikrokerne oder zeigte Zytotoxizität.

Zusammenfassend lässt sich somit sagen, dass mehrere urämische Toxine *in vitro* eine genotoxische Wirkung entfalten, allerdings erst in Konzentrationen die in Patienten nicht erreicht werden. Natürlich lässt sich nicht ausschließen, dass diese durch die chronische Exposition in sensitiven Geweben oder Zelltypen schon bei physiologisch relevanten Konzentrationen auftreten könnten. Wahrscheinlicher ist jedoch, dass das Problem der erhöhten Genomschäden multifaktoriell ist. Neben der eventuell sogar synergistischen Wirkung der urämischen Toxine, spielen vermutlich auch noch folgende Faktoren eine Rolle: (1.) vermehrte Sauerstoffradikalenbildung durch Inkompatibilität zwischen Dialysemembranen und Blut, (2.) verschlechterte DNA-Reparatur der Dialysepatienten (3.) ein geschwächtes Immunsystem sowie (4.) ein geschwächtes Antioxidanzsystem.

G Summary

In patients suffering from end-stage renal disease who are treated by hemodialysis genomic damage as well as cancer incidence is elevated.

One possible cause for the increased genomic damage could be the accumulation of genotoxic substances in the blood of patients. Two possible sources for those toxins have to be considered. The first possibility is that substances from dialysers, the blood tubing system or even contaminated dialysis solutions may leach into the blood of the patients during dialysis. Secondly, the loss of renal filtration leads to an accumulation of substances which are normally excreted by the kidney. If those substances possess toxic potential, they are called uremic toxins. Several of these uremic toxins are potentially genotoxic.

Within this thesis several exemplary uremic toxins have been tested for genotoxic effects. Additionally, it was analysed whether substances are leaching from dialysers or blood tubing and whether they cause effects in *in vitro* toxicity testing. The focus of chemical analytisis was on bisphenol A (BPA), the main component of plastics used in dialysers and dialyser membranes, as well as on di(ethylhexyl)phthalate (DEHP), which is used as plasticiser in many blood tubing systems.

For this purpose dialysis was simulated using five different kinds of dialysers and PVC blood tubing. Two different eluents (reverse osmotic water and 17.2% ethanol) were used while temperature, dialysis period and flow rate conformed to the real dialysis modalities. The eluates obtained were analysed by LC-MS/MS for their BPA as well as DEHP content. The DEHP concentration did not exceed the limit of quantification in any eluate. In contrast, all of the eluates contained quantifiable amounts of BPA.

From these results it was extrapolated that 6.4 ng to 4.3 µg BPA are leaching per dialyser. The amount of BPA depended on the duration of dialysis, the type of dialyser and the eluent. The amount of leaching BPA was higher when 17.2% ethanol was used. This is an eluent which has similar extraction properties as blood. Additionally substantial differences between different batches of dialysers could be detected. Assuming the worst case –we estimated the additional body burden of BPA to be 0.84 ng/ml blood (5 l blood) or 61.4 ng/ kg body weight (70 kg person) per dialysis session.

In order to estimate the potential risk for the patient, these values had to be compared to the plasma levels of average humans. Those values are between 0.3 – 18.9 ng/ml blood. One also has to take into consideration that BPA has no tendency for bioaccumulation and a short half-life (< 1 day). Therefore the additional burden due to dialysis is in the low range of the average body burden. Furthermore, the additional body burden of 61.4 ng/ kg body weight is still a factor of more than 800 below the threshold of the European Food Safety Agency (EFSA) for the tolerable daily oral intake (50 µg/kg bw).

However, the eluates contained additional substances which have not been identified in this study. For this reason a potential risk for the patient could not initially be ruled out. As a consequence *in vitro* cytotoxicity and genotoxicity tests were conducted. The concentration of eluates in cell culture media was 25 (ethanol eluates) to 50 times (water eluates) higher than the one reached in blood. No evidence for cytotoxicity or genotoxicity could be found in any of the tests. Solely the test for estrogenic activity yielded slightly positive results when performed with the ethanol eluates. As BPA possesses estrogenic activity, comparative experiments with pure BPA were conducted. BPA showed a statistically significant estrogenic effect at 7.2 ng/ml, while the BPA concentration in the cell culture media was between 0.03 ng/ml and 5.8 ng/ml. The estrogenic activity of the eluates could therefore not have been caused by BPA exclusively. Despite the slight estrogenic activity, the risk for the hemodialysis patient by leaching substances can be regarded as negligible. Although it is known that xenoestrogens may contribute to tumour development, an increased cancer incidence of hormone responsive tissue - like breast tissue - would be expected. In dialysis patients, this is not the case.

The second part of the work focused on genotoxic testing of exemplary uremic toxins. At the moment more than 90 different toxins are known, therefore the first task was to choose potentially genotoxic ones. Most of the work focused on homocysteine, an intermediate of the methionine cycle. Elevated plasma levels of homocysteine have been correlated to an increased micronucleus frequency in healthy persons. Micronuclei are markers for genomic damage. This correlation was confirmed by *in vitro* tests with several cell lines during this study. Exposure to homocysteine resulted in an increased micronucleus frequency. However, a homocysteine concentration of 3 mM was necessary to induce micronuclei *in vitro*,

while the plasma concentration of homocysteine in humans exceeds 100 µM only in very severe cases. In the comet assay (a second genotoxicity test) no genomic damage could be observed up to cytotoxic concentrations.

Frequently, micronuclei result from disturbances of the spindle apparatus. These often lead to disturbances in the cell cycle. In fact, incubation with homocysteine did increase the percentage of cells in S-phase. However, this was not the case until a concentration of 5 mM homocysteine was reached in the cell culture medium. An increased plasma level of homocysteine has also been correlated to a decreased overall DNA cytosine methylation. Therefore we analysed whether changes of DNA cytosine methylation are responsible for the extension of the S-phase. However, Hcy concentrations of up to 5 mM did not change levels of DNA methylation. Higher concentrations were not evaluated because they were cytotoxic.

Another common reason for increased genomic damage is the formation of free radicals. However, addition of the radical scavenger N-acetylcysteine did not reduce the micronucleus frequency. Flow cytometric measurements of reactive oxygen species failed to detect oxidative stress due to Hcy. On the contrary, radical stress induced by hydrogen peroxide could be reduced by co-incubation with homocysteine. This was probably due to the intracellular conversion of homocysteine to the intracellular antioxidant glutathione. Therefore oxidative stress can be ruled out as cause for micronuclei.

Additionally, micronucleus tests with the derivate of homocysteine: homocysteine-thiolactone have been performed. If homocysteine wrongly enters the biosynthetic apparatus instead of methionine, it is activated and subsequently converted to homocysteine-thiolactone. This explains the increasing level of homocysteine-thiolactone if the homocysteine level in the cell increases. Incubation of cells with 1 mM homocysteine-thiolactone increased the micronucleus frequency significantly. However, this concentration was also cytotoxic which prevented the unambiguous proof of direct genotoxicity.

Further uremic toxins of interest are the so-called advanced glycation end-products. They result from non-enzymatic reaction of reducing sugars with free amino groups of peptides and proteins and are a heterogenic group of proteins. Two exemplary representatives of this group have been synthesized and tested in the comet assay. They induced genotoxicity although the severity was depending on the batch.

Finally, the toxicity of the potential uremic toxin leptin was evaluated. Leptin is a hormone which is mainly synthesized by adipocytes and which regulates food intake and energy expenditure. Starting at 1 µg/ml leptin, induced genomic damage detectable by comet assay. It did not induce micronuclei or cytotoxicity.

To summarize: several uremic toxins exhibit genotoxicity *in vitro* but only at concentrations higher than those reached in the patient. Still it cannot be ruled out that chronic exposure of sensitive tissue or cell types may have a genotoxic effect at physiologically relevant concentrations. It is more likely that this problem is multifactorial. Apart from possible synergistic effects of uremic toxins, other factors are probably involved: (1.) increased formation of reactive oxygen species due to incompatibility of dialysis membranes and blood, (2.) an impaired DNA repair system of dialysis patients, (3.) a weakened immune system, or (4.) an reduced antioxidant defence.

H Acknowledgements

Foremost I would like to thank my supervisor Prof. Dr. Helga Stopper for her active interest in my work, her consistently great support and her expert guidance.

I warmly thank Prof. Dr. Vienken and Prof. Dr. Heidland for their fruitful discussion about my work and their excellent advice.

My thanks go also to Prof. Dr. Benz and Prof. Dr. Nieswand for supervising my work on behalf of the Faculty of Biology and the International Graduate School of the University of Würzburg.

I would like to thank the past and the present members of the Stopper lab for providing a great work environment, sharing the ups and down of a grad student's life and for their help and chats.

Special thanks to Judith Manz and Theresa Ehrlich for their assistance with the laboratory work.

I also owe a lot of thanks to Andreas Brink, Eva Trösken, Nataly Bittner and Wolfgang Völkel for their help with mass-spectrometry.

I am especially grateful to Cristina Bouchcinsky for reading my manuscript

This research has been founded by Fresenius medical care. I gratefully acknowledge this support.

Finally, I want to thank Reinhard Fink for his loving support and his patience during the last stages of my PhD.

I Appendix

1 List of Abbreviations

2-EH	2-Ethylhexanol
5-Aza-C	5-Aza-cytidine
5cx-MEPP	Mono-[2-ethyl-5-carboxypentyl] phthalate
5cx-MMHP	Mono-[2-(carboxymethyl)hexyl] phthalate
5-mdCyd	5-Methyl-2`deoxycytidine
5OH-MEHP	Mono-[2-ethyl-5-hydroxylhexyl] phthalate
5oxo-MEHP	Mono-[2-ethyl-5oxylhexyl] phthalate
7-AAD	7-Aminoactinomycin
8-OHdG	8-Hydroxy-2-deoxy-Guanosin
AARS	Aminoacryl-tRNA
AGE	Advanced glycation end-product
AH	Adenosyl-homocysteinase
AMP	Adenosine monophosphate
AMU	Atomic mass unit
ATP	Adenosine triphosphate
ATSDR	Agency for Toxic Substances and Disease Registry
BHMT	Betaine-homocysteine-S-methyltransferase
BMI	Body mass index
BPA	Bisphenol A
BrdU	5-Bromo-2-deoxyuridine
BSA	Bovine serum albumine
BW	Body weight
CA	Comet assay
CBS	Cystathione-β-synthase
CIMS	Chronic inflammation malnutrition syndrom
CML	Carboxy(methyl)lysine
cps	Counts per second
CRF	Chronic renal failure
Cyt B	Cytochalasin B
DCF	2`7`-Dichlorofluorescein
DCFH-DA	2`7`-Dichlorofluorescein diacetat
ddH_2O	Double-distilled water, HPLC-grade water
DEHP	Di-ethylhexyl-phthalate
dGuo	2`-Deoxyguanosine
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DOP	Dioctyl phthtalate
DTNB	5`5`-Dithiobis-2-nitrobenzoic acid
ECB	European Chemical Bureau
EDTA	Ethylene-diamine-tetraacetic acid
EFSA	European Food Safety Agency
ELISA	Enzyme-linked ImmunoSorbent Assay

EPA	European Protection Agency
ER	Estrogen Receptor
ESRD	End-stage renal disease
EtOH	Ethanol
FACS	Fluorescence-activated cell-sorting
FBS	Fetal bovine serum
FIGE	Field inversion gel electrophoresis
FITC	Fluorescein isothiocyanate
FSC	Forward scatter
FSH	Follicle stimulating hormone
g	Gram
g	Gravitational constant
GC-MS	Gass-chromatography-mass spectrometry
GFR	Glomerular filtration rate
GSH	Glutathione (reduced form)
GSSG	Glutathione (oxidized form)
h	Hours
HCl	Hydrochloric acid
Hcy	Homocysteine
Hcy-T	Homocysteine-thiolactone
HD	Hemodialysis
HDF	Hemodiafiltration
HDL	High-density lipoproteins
HEPES	4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid
HPLC	High-performance liquid chromatography
HPLC-MS/MS	HPLC linked to a tandem-mass spectrometer
HPRT	Hypoxanthine-guanine phosphoriboxyl transferase
HUVEC	Human umbilical vein endothelial cells
l	Liter
LC-MS	Liquid chromatography-mass spetrometry
LC-MS/MS	Liquid chromatography-tandem-mass spectrometer
LD_{50}	Leathal dose, 50%
LDL	Low-density lipoprotein
LMP-Agarosis	Low melting point agarosis
LOAEL	Lowest observed adverse effect level
LOD	Limit of detection
LOEL	Lowest observed effect level
JAK	Janus kinase
M	Mole
m/z	Mass-charge-ratio
mA	Milli Ampere
MAT	Methionine-adenosyltransferase
MEHP	Mono(2-ethylhexyl)phthalate
MET	Methionine
MGO	Methylglyoxal
min	Minutes
MMC	Mitomycin C
MMS	Methylmethane sulfonate

MN	Micronuclei
mRNA	Messenger ribonucleic acid
MS	Mass spectrometry
MS/MS	Tandem mass spectrometry
MW	Molecular weight
µg	Microgram
µl	Microliter
µM	Micromolar
n	Normal
NAC	N-acetylcysteine
$NaCNBH_3$	Sodium cyanoborohydride
$NADP^+$	Nicotinamide adenine dinucleotide phosphate (oxidized form)
NADPH	Nicotinamide adenine dinucleotide phosphate (reduced form)
NaOH	Sodium hydroxide
NF-κB	Nuclear factor-kappa B
NOEL	No observed effect level
p	Probability
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate-buffered saline
PBS-CMF	Phosphate buffered saline – calcium and magnesium free
PD	Peritoneal dialysis
PI	Propidium iodide
Ppar-α	Peroxisome proliferators-activated receptor α
P/S	Penicillin / streptomycine
PS	Horse serum
PVC	Polyvinyl chloride
RAGE	Receptor for advanced glycation end products
RNA	Ribonucleic acid
ROS	Reactive oxygen species
sec	Seconds
S.D.	Standard Deviation
SAH	S-adenosylhomocysteine
SAM	S-adenosylmethionine
SIR	Standardized incidence ratio
SSC	Side scatter
STAT	Signal transducers and activators of transcription
TDI	Tolerable daily intake
tHcy	Total homocysteine
THF	Tetrahydrofolate
TRIS	Trishydroxymethylaminomethane
tRNA	Transfer RNA
PVP	Polyvinylpyrrolidon

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5 Curriculum vitae

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Current employment

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06/2004 - 12/2006	Associated member of the International Graduate College "Target Proteins"
10/1997 - 07/2003	Undergraduate study of biology, University of Konstanz Degree: Diploma in biology
08/2000 - 06/2001	Undergraduate study of biology, University of Guelph, Canada
10/1996 - 09/1997	Undergraduate study of chemistry at the Albert-Ludwigs University, Freiburg
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6 Publications

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7 Ehrenwörtliche Erklärung

Hiermit erkläre ich, dass ich die Arbeit selbständig verfasst und keine anderen als die von mir angegebenen Quellen und Hilfsmittel benutzt habe.

Ferner erkläre ich, dass ich nicht versucht habe, diese Dissertation anderweitig mit oder ohne Erfolg in gleicher oder ähnlicher Form einzureichen.

Ich habe keine Doktorprüfung an einer anderen Hochschule abgelegt oder endgültig nicht bestanden.

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