The biological significance of chemically-induced DNA adducts in relation to background DNA damage

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1 Introduction

1.1 Chemical carcinogenesis

Chemical carcinogenesis is a complex multistep process by which chemical carcinogens induce tumor formation. The initial carcinogen-induced events typically include DNA damage that is the result of covalent binding of carcinogens to DNA (DNA-adduct formation) [1,2], and of other modifications to DNA, such as oxidative damage or alterations to DNA structure like DNA-strand breakage, crosslinking, chromosomal rearrangements or deletions [3]. Although cells posses mechanisms to repair many types of DNA damage [4], these are not always entirely effective, and residual DNA damage can lead to mutations e.g. in oncogenes, tumor-suppressor genes or in genes that control the cell cycle (tumor initiation). These mutations can result in a clonal cell population with a proliferative or survival advantage that can be expanded (tumor promotion). Additional mutagenic mechanisms responsible for the transition to malignancy and further to metastasis are summarized as tumor progression [3,5]. The development of a tumor requires many such events, occurring over a long period of time. For this reason human cancer induction often takes place within the context of chronic exposure to chemical carcinogens.

A well-known example of this is the association of long-term smoking of tobacco with increased risk to develop lung cancer [6].

For classification reasons, carcinogenic compounds are often divided into subgroups. For example into genotoxic or non-genotoxic carcinogens (based on their effect in *in vitro* genotoxicity assays), or into DNA-reactive and non-DNA target carcinogens (based on their potential to interact with DNA). However, these classifications do not take into account that most carcinogens exert a variety of events, involving DNA reactive and non-reactive as well as genotoxic, non-genotoxic actions or epigenetic events [7]. Also the terms "tumor initiation, promotion and progression" are descriptive terms and the underlying mechanisms are definitely manifold and often unknown. Originally, tumor initiation by genotoxic agents and tumor promotion by non-genotoxic agents were regarded as separate categories [5]. This conceptual distinction may not be maintained and is currently refined. Although, the various mechanisms of tumor promotion might be typically non-genotoxic, some agents may elicit genotoxicity along with tumor promoting properties and it is misleading to conclude that the terms "non-genotoxic carcinogen" and "promoter" are synonyms. The mechanisms underlying tumor initiation and progression as well include both genotoxic and non-genotoxic events [3].

With regard to the risk assessment of chemical carcinogens, however, the distinction between genotoxic and non-genotoxic mechanisms is of importance, since this influences the procedure used to extrapolate results from high-dose exposure in animal bioassays to low-dose exposure relevant in humans. In principle non-genotoxic carcinogens are regulated using the no observed effect level (NOEL) and safety factors, whereas for genotoxic carcinogens the default procedure for extrapolation to low-dose is linear. This is based on the assumption that any small increase by exposure to an exogenous genotoxic agent may contribute to the process of carcinogenesis by a nonzero increment [8,9].

1.2 Genotoxicity

The term genotoxicity is broadly used for all lesions in the genetic material. The genetic modifications might be spontaneous (chemical instability of DNA, depurinations) or induced (DNA adducts, chromosomal aberrations, mutations) and are not only associated with the initiation of cancer but also with other diseases like

e.g. atherosclerosis or progeroid syndroms. A large number of endpoints, evaluating different genetic alterations have been developed and established for genotoxicity testing of chemicals. Depending on the specific type or class of genetic change investigated (e.g. DNA-adduct formation, DNA strand breaks, chromosomal aberrations, gene mutations, etc.), the endpoints have different implications and relevance for the process of tumorigenesis.

1.2.1 DNA adducts

DNA adducts are primary lesions formed by electrophilic genotoxic agents by covalent binding to DNA. The purine and pyrimidine bases guanine, adenine, thymine and cytosine contain numerous nucleophilic sites. Hence, DNA is prone to alterations by electrophils. DNA-adduct formation is generally considered as an important step in chemical carcinogenesis, because for the majority of chemical carcinogens DNA-adduct formation has been demonstrated (e.g. aflatoxins, polycyclic aromatic hydrocarbons, alkylating agents). Many of these carcinogens are not electrophilic initially, but require metabolic activation to the ultimate DNA-binding carcinogen, e.g. the generation of DNA reactive epoxides by cytochrome P450 dependent monooxygenases. Well studied examples are the epoxide derivatives of benzo(a)pyrene or dimethyl(a)anthracene that attack the nitrogen in position 2 of guanine and form adducts that could be linked to carcinogenesis [10] long time ago. However, DNA adducts are primary lesions and are not equivalent to mutations or cancer. If and to what extent DNA adducts are processed into biologically relevant lesions depends on various factors like the chemical structure, miscoding properties or DNA repair processes. DNA reactive carcinogens usually result in multiple adducts. For instance methylating agents like methyl methanesulfonate can add methyl groups to DNA bases at about one dozen of different sites in DNA bases [11]. Different adducts have different consequences. Some adducts are highly promutagenic (e.g. alkylations at O^6 of guanine) and can change the coding properties of DNA bases by influencing the formation of van der Waals interactions [12]. This can result in mismatches during DNA replication (Fig. 1).

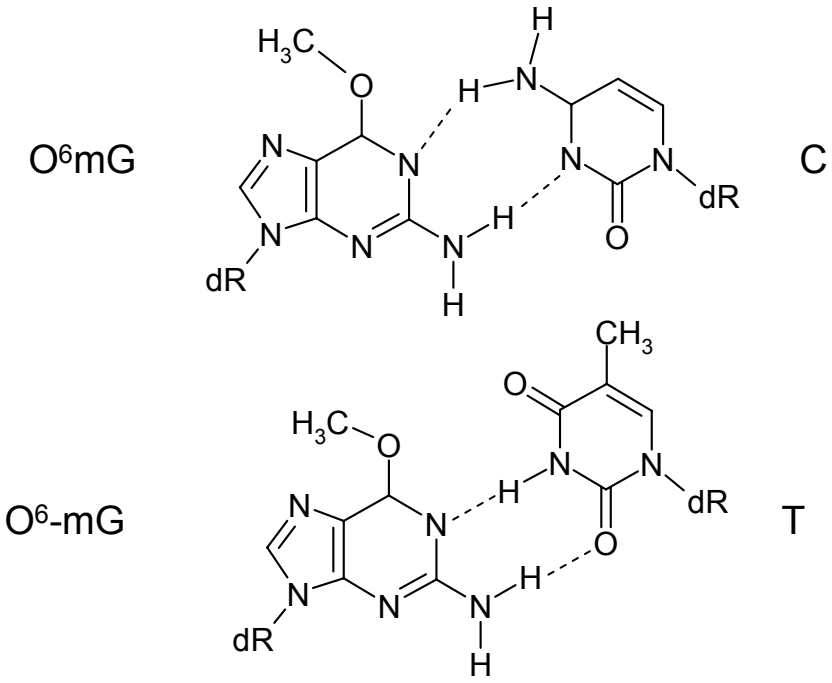


Fig. 1. Two possible structures of O^6-methylguanine (O^6-mG) base pairs. (A) *O*^{6}-mG wobble base pair with cytosine. (B) *O*^{6}-mG mispair with thymine instead of cytosine. During DNA replication the methyl group in position O^6 of guanine enhances the probability of thymine incorporation instead of cytosine [12].

If not removed by mismatch repair, after one more round of replication this could results in heritable mutations (Fig. 2). These mutations must not necessarily be in critical regions of the genome or might induce cell death, but if occurring in critical genes (e.g. oncogenes, tumor suppressor genes or genes of cell cycle control) this may result in initiation of cells providing a proliferative or survival advantage compared to normal cells. Some DNA adducts have poor miscoding properties and are therefore less promutagenic, but may facilitate depurination (e.g. methylations at nitrogen 7 of guanine), and induce apurinic sites which might give rise to mutations, because DNA polymerases preferably incorporate adenosine opposite to a non-instructive apurinic site, if passing by this lesion [13,14].

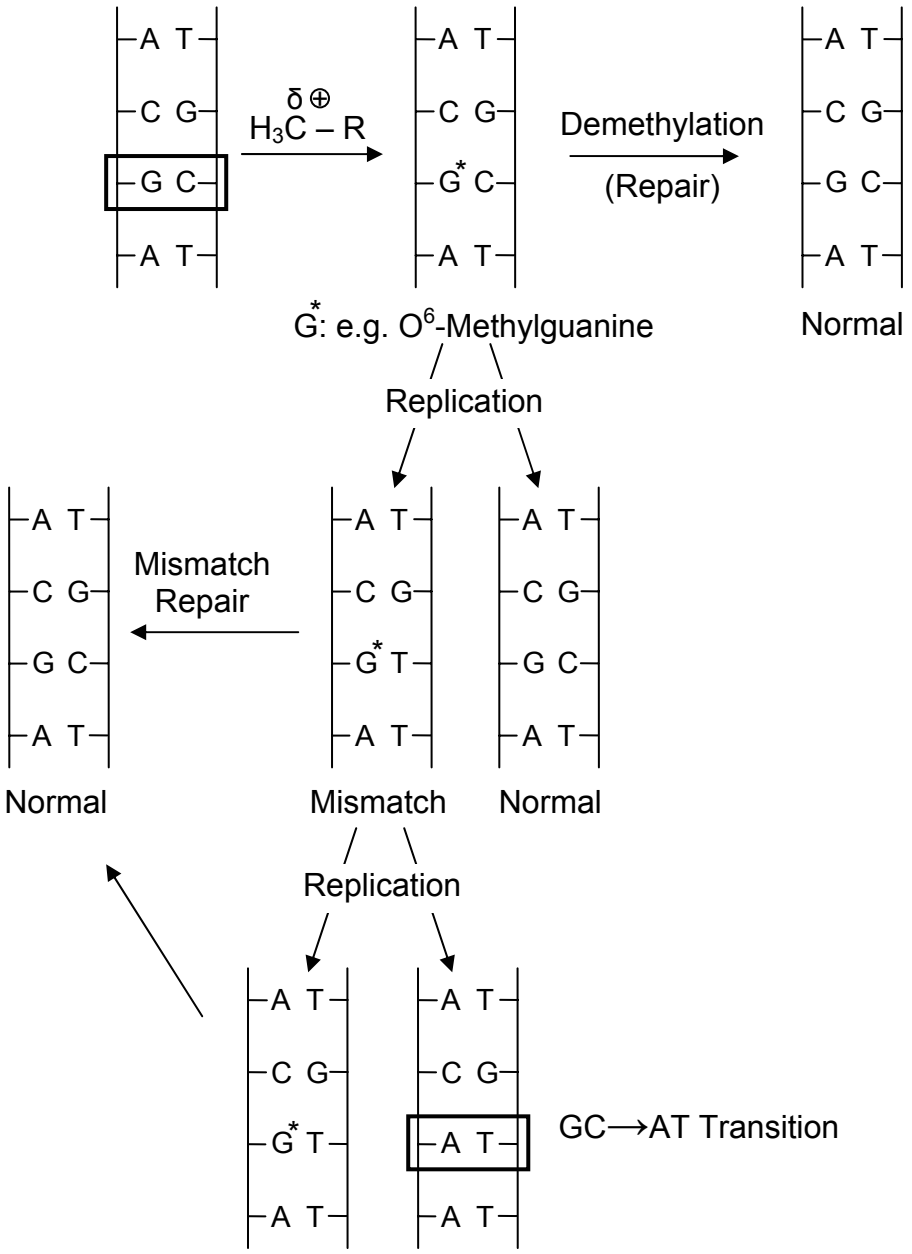


Fig. 2. From DNA adduct to base pair substitution mutation. The possible pathway from a methylation at position O^6 of guanine, if mispairing with thymine (T) occurs during replication results after two rounds of replication in a GC-AT transition mutation [15].

As diverse DNA adducts and their consequences might be, as manifold are the cellular mechanisms that have evolved to maintain genome integrity and to reduce the probability of DNA adducts being fixed into biologically relevant lesions [4]. Efficient DNA repair mechanisms process both the adduct itself and secondary damage induced by adducts (e.g. apurinic sites, DNA strand breaks), by e.g. direct reversal (e.g. demethylation), base excision repair, nucleotide excision repair, mismatch repair and recombinant repair [16]. Additionally, effects on cell cycle delay

or cell death by apoptosis or necrosis influence the translation of DNA adducts into cellular transformations.

The biological significance of DNA adducts could be based on their contribution to subsequent manifestations of cellular transformations which are relevant in the process of tumorigenesis. In this context, the evaluation of the quantitative relationship between chemically-induced DNA adducts and the appearance of subsequent molecular or genetic alterations – like DNA strand breaks, chromosomal damage or mutations – could be a first step to characterize the relevance of DNA adducts for a biologically significant outcome.

1.2.2 Endogenous DNA damage

DNA reactive agents are not only of exogenous origin but are also produced endogenously. Numerous endogenous agents and actions were characterized that contribute permanently to a background level of DNA adducts [17]. Background levels have been estimated to be on the order of 1 alteration in 10^5 normal nucleotides [18]. Major contributions were identified to involve DNA alkylations, direct oxidation of DNA and reaction of DNA with lipid peroxidation products [17].

An agent probably responsible for endogenous aberrant DNA methylation is S-adenosyl-L-methionine (SAM). SAM is a methyl-donor in numerous biochemical enzymatic reactions and highly abundant in cells and induces (non enzymatic) methylations in DNA mainly at the nitrogen 7 of guanine, 3 of adenosine and to a small extent at O^6 of guanine [19]. Since O^6-methylguanine is a highly mutagenic lesion, consequently SAM might contribute to spontaneous mutagenesis, representing an endogenous mutagen.

One major source of endogenous background DNA lesions, however, are reactive oxygen species (ROS) and products of lipid peroxidation [17,20]. Numerous different oxidative DNA adducts have been identified, for which promutagenic properties have been demonstrated [21,22]. The identity of the oxidants causing these lesions however is manifold and has not been elucidated completely. A general process is the 1-electron reduction of O_2 by cellular metabolism (cytochrom c oxidase) or by NADPH oxidases in macrophages that gives rise to the superoxide radical anion ($O_2^{\bullet-}$). This process is not only fundamental for the energy production by aerobic organisms but also for the respiratory burst of macrophages. Another important radical produced endogenously by macrophages is nitric oxide ($NO^{\bullet}$) which

readily reacts with superoxide forming a reactive nitrating and oxidizing species, peroxynitrite ($ONOO^-$). To prevent themselves of oxidative damage aerobic organisms generally possess enzymatic detoxification systems. It involves dismutation of two $O_2^{\bullet-}$ molecules to hydrogen peroxide and molecular oxygen by superoxide dismutase [17]. The superoxide radical anion and hydrogen peroxide (H_2O_2) are only weakly reactive [23]. Problematic is the Fenton reaction of H_2O_2 to the hydroxyl radical ($HO^\bullet$) which is catalyzed by iron or copper ions in the presence of a reducing agent such as the $O_2^{\bullet-}$. The highly reactive hydroxyl radical readily reacts with DNA bases directly or oxidize macromolecules (e.g. lipids) to generate reactive intermediates that may form covalent DNA adducts, e.g. 4-hydroxy-2-nonenal.

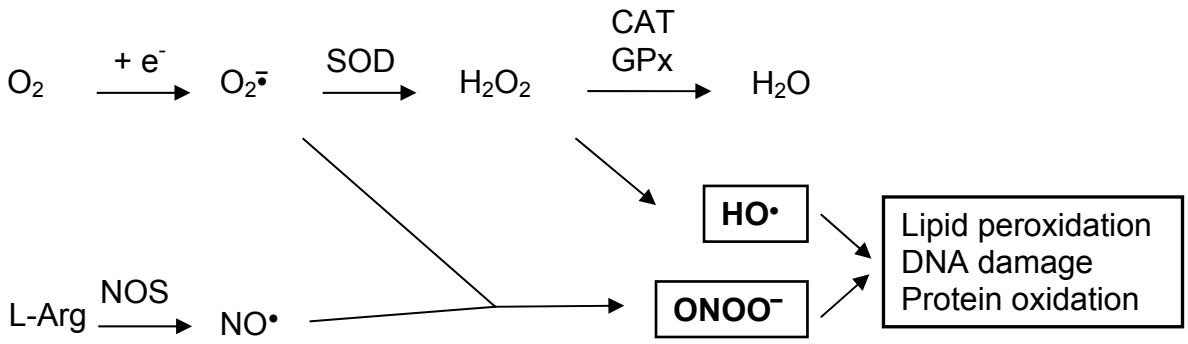


Fig. 3. Oxygen (O_2) can be reduced to superoxide ($O_2^{\bullet-}$) by different cellular mechanisms. $O_2^{\bullet-}$ can be reduced to hydrogen peroxide (H_2O_2) by superoxide dismutase (SOD). H_2O_2 is reduced to water either by glutathione peroxidase (GPx) using glutathione as substrate or by catalase (CAT). In the presence of e.g. free iron, H_2O_2 undergoes the Fenton reaction to form the hydroxyl radical ($HO^\bullet$). When nitrogen monoxide ($NO^\bullet$) is formed e.g. from L-Arginine (L-Arg) by NO synthase (NOS), $O_2^{\bullet-}$ could react with $NO^\bullet$ to form peroxynitrite ($ONOO^-$). Both $HO^\bullet$ and $ONOO^-$ are very reactive and can modify cellular macromolecules. The less-reactive molecules $O_2^{\bullet-}$ and H_2O_2 can serve as cellular signaling molecules. Modified from [24,25].

1.2.3 Measurement of DNA adducts

The currently most frequently used approaches for DNA-adduct analyses are mainly based on ^{32}P postlabeling, immunoassays and mass spectrometry. These approaches differ greatly regarding sensitivity and specificity. High sensitivity is provided by ^{32}P postlabeling methods (about 1 adduct in 10^9 normal nucleotides). These methods are based on DNA digestion to 3′-phosphates, followed by adduct enrichment and 5′-radiolabeling of the adducts with high specific activity ^{32}P from γ^{32}P-ATP by T4 polynucleotide kinase. Then the labeled bis-phosphates can be separated and analyzed on thin layer chromatography (TLC) or high pressure liquid chromatography (HPLC). Requiring only small quantities of DNA (1 to 10 µg) ^{32}P postlabeling methods are still widely used in molecular epidemiology, where the amount of DNA often is limited. Main weaknesses of the ^{32}P postlabeling methods are low specificity and limited reproducibility, as well as being work intensive and the need to handle radioactive ^{32}P. In particular for "low molecular weight" adducts (e.g. O^6mdGuo, 8-oxodGuo) it is difficult to achieve accurate and precise measurements using the adduct enrichment methods available – e.g., immunoaffinity, nuclease P1 enrichment or butanol extraction [26,27].

Immunoassays apply antibodies against specific DNA adducts or modifications in DNA being also valuable for visualizations of DNA adducts e.g. in intact nuclei. The structure identification depends on the specificity of the antibody and accurate and precise quantifications might be difficult due to cross-reactions of antibodies with structure-related adducts.

A special type of MS based approach is the use of accelerator mass spectrometry (AMS) [28]. AMS represents the most sensitive analytical method available to date for detecting DNA adducts with a limit of detection that may be as low as 1 adduct per 10^{11} unmodified DNA bases [29]. However, it is limited to the detection of radiolabeled compounds (usually ^{14}C). To detect ^{14}C radioisotope incorporation into DNA, the sample is converted to graphite prior to introduction into the accelerator mass spectrometer [30]. The disadvantages of AMS are the need for ^{14}C-radiolabeled chemicals, the lack of structural information, and the possible biosynthetic incorporation of ^{14}C into DNA.

Other methods based on mass spectrometry are able to identify adducts based on molecular weight and are continually becoming more sensitive because of significant technological improvements in recent years. The earlier assays employing

mass spectrometry for adduct analyses were mostly based on the detection of the adducted base or of the carcinogen residue itself (after hydrolysis of the adducts). It often included chemical derivatisation and gas chromatography coupled to mass spectrometry [31]. Liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) using "soft" ionization by an electrospray interface is an alternative to analyze DNA-adducts either as 2′-deoxyribonucleosides [32-35] or as base adducts [36]. Analyzing 2′-deoxyribonucleosides has the advantage of excluding potential contamination of DNA by RNA. The use of tandem mass spectrometers with multiple reaction monitoring (MRM) mode offers high sensitivity paired with high selectivity.

A particular challenge of DNA-adduct detection with LC-MS/MS is the determination of background levels. The reason for that is that the levels of these adducts are very low and that they are embedded in a high excess of matrix of unmodified normal nucleotides. Therefore, typically a sample clean up is required after hydrolysis of the DNA. These procedures are prone to sample loss, and also in the case of 8-oxodGuo to artifact formation.

1.2.4 Markers of genotoxicity

DNA adducts are primary lesions which contain information on the genotoxic agent involved and levels of DNA adducts indicate the biologically effective dose that has reached the DNA of cells or tissue under study. Adduct data provide valuable information for molecular epidemiological studies giving an estimation of exposure. Together with other primary (repairable) lesions such as DNA stand breaks DNA adducts are not necessarily inheritable cellular alterations (Fig. 4).

Specific DNA adducts contain information on the carcinogen involved, as opposed to other endpoints of genotoxicity (DNA strand breaks, chromosomal damage, gene mutations) that no longer allow to identify the DNA reactive agent or genotoxic process. DNA strand breaks can be induced by DNA adducts via apurinic sites. Apurinic sites are sensitive to hydrolysis and can result in strand breaks even under cellular pH conditions. Strand breaks also occur spontaneously or in the sequence of base excision repair processes on DNA adducts, if e.g. ligation of the repaired DNA strands fails.

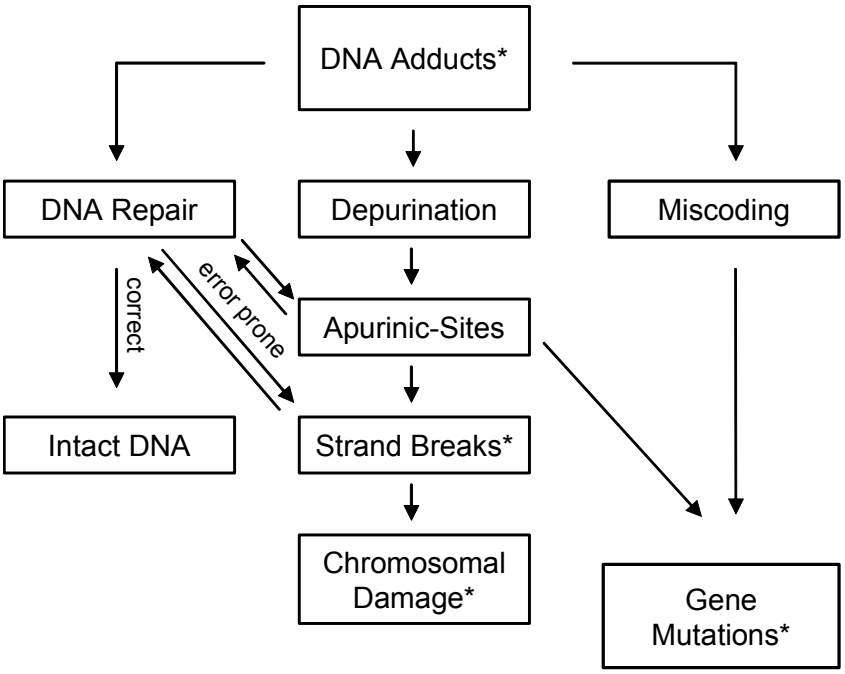


Fig. 4. DNA adducts represent the initial interaction of genotoxic carcinogens with the target DNA and their fate determines their biological significance for the process of tumorigenesis. The asterixes indicate the endpoints measured in this work.

One method widely used to assess DNA strand breaks is the comet assay (also called single-cell gel electrophoresis). The principle of the comet assay is the migration of DNA fragments into a agarose gel during electrophoresis. In contrast to undamaged nuclear DNA the small DNA fragments migrate into the gel forming a "tail" of a "comet" and can be analyzed after DNA staining. Under alkaline conditions also alkali-labile sites (e.g. abasic sites) are detected after being converted into DNA strand breaks during incubation in the electrophoresis buffer. The extent of DNA migrated into the tail is used to quantify DNA damage. This assay is widely used as an indicator of genotoxicity. Since DNA strand breaks are not only the result of genotoxic interaction of chemicals, but might also occur during necrosis or apoptosis, cell death induced by the chemical under study might constitute a bias for the interpretation of the results.

A genotoxicity endpoint widely used to assess chromosomal damage *in vivo* and *in vitro* is the micronucleus test (also known as micronucleus frequency assay) [37].

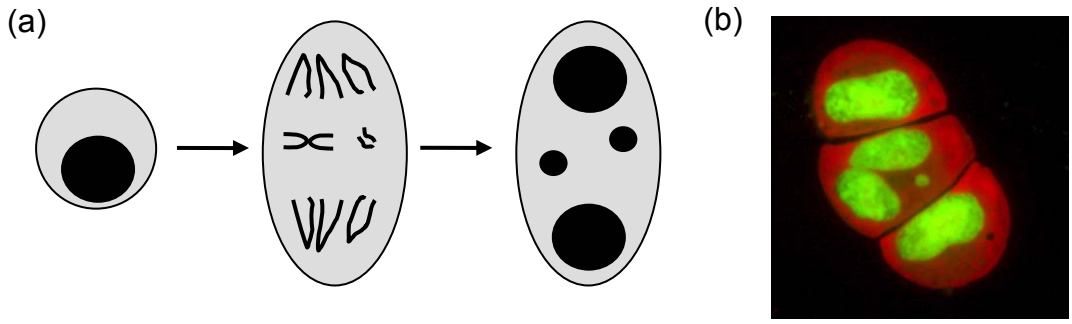


Fig. 5. (a) Schematic diagram showing the origin of micronuclei from either a lagging chromosome fragment or a whole chromosome (adapted from [38]) (b) Image of L5178Y mouse lymphoma cells stained with acridine orange. The cells were incubated with cytochalasin B. This prevents cytokinesis resulting in a binucleated cell after one nuclear division.

The micronucleus test detects different kinds of chromosomal damage induced by various mechanisms [39]. Micronuclei are generated during cell division if chromosomal fragments or whole chromosomes that are not attached to the spindle organelles are excluded from the daughter nuclei and form extra "nuclei" within the cytoplasm. The formation of micronuclei in dividing cells can also be the result of not or mis-repaired DNA adducts. The induction of micronuclei by an agent could be considered a biological relevant effect because the relation between micronuclei and carcinogenesis is strongly supported. This is based on the observation that many mechanisms involved in micronuclei formation (clastogenicity, aneuploidy, chromosomal rearrangements, changes in gene expression, altered DNA repair to name only the most important) are all in all effects that enhance genomic instability associated with tumorigenesis. Further, the results from a recently published prospective cohort study within a population of healthy subjects provided evidence that micronuclei frequency in peripheral blood lymphocytes is a predictive biomarker for cancer risk [38].

The induction of gene mutations mediated by DNA adducts as heritable alterations could be considered an endpoint of major importance in the assessment of the biological significance of DNA adducts. To quantify induced gene mutations (mutant frequency) *in vitro*, the L5178Y mouse lymphoma thymidine kinase mutation assay can be used. This mammalian cell mutation test (also known as the mouse lymphoma assay) is part of the recommended test battery for genotoxicity testing of

chemicals and an OECD guideline on this test exists [40]. The used mouse lymphoma cell line (L5178Y $tk^{+/-}$ 3.7.2C) is heterozygous at the thymidine kinase locus (TK) on chromosome 11. Inactivating the tk^{+} allele induces trifluorothymidine (TFT) resistance, and $tk^{-/-}$ mutants can be selected for in a background of $tk^{+/-}$ non-mutant cells. The mouse lymphoma thymidine kinase mutation assay can detect a variety of mutations, including point mutations and small mutations within the TK locus, losses of the whole functional allele, larger deletions including TK and cytogenetically detectable chromosome aberrations such as translocations [41-43].

1.3 Chemically-induced hepatocarcinogenesis

Hepatocellular carcinoma is an extremely malignant disease usually associated with extremely poor prognosis. Manifold molecular variants of hepatocellular carcinomas were identified. High incidences are found in sub-Saharan Africa and eastern Asia, areas associated with three major risk factors (80% of all cases). High prevalence of chronic hepatitis B and C and with high levels of aflatoxin B1 in food [44]. In areas with low hepatitis virus prevalence (e.g. Germany), incidence of hepatocellular carcinoma is lower but ethanol induced liver cirrhosis constitutes an important risk factor to develop hepatocellular carcinoma [45]. Even though the etiology of this disease is well characterized the mechanisms involved from initiation, promotion and progression of malignant phenotypes are not elucidated completely. Prior to the appearance of hepatocellular carcinoma liver cirrhosis is frequently observed as a process associated with high levels of cytotoxic damage in hepatocytes but also with inflammation and oxidative stress. Hence, not only hepatocytes are proposed to be involved in the initiation and progression of hepatocellular carcinoma, but also inflammatory cells (e.g., macrophages or neutrophils). Kupffer cells are the macrophages of the liver and are important in the regulation of inflammatory events as well as the elimination of pathogens. Activation of Kupffer cells results in the release of (nonspecific) proinflammatory, and cytotoxic mediators like cytokines (TNFa) or superoxide.

1.4 Kupffer cells and background DNA damage

Kupffer cells are the macrophages of the liver involved in host defense and mediation of inflammation. The superoxide radical anion ($O_2^{\bullet-}$) is produced in Kupffer cells from oxygen (O_2) and NADPH catalyzed by the phagocyte NADPH oxidase (PHOX). The complex of PHOX is formed by five major subunits, i.e. two membrane-bound proteins p22phox and gp91phox, and three cytosolic proteins p40phox, p47phox and p67phox. The subunit p47phox is required for the assembly and function of PHOX. Lack of the p47phox gene abolishes $O_2^{\bullet-}$ formation in macrophages [46,47].

Patients deficient in p47phox have chronic granulomatous disease. Their neutrophils are unable to produce $O_2^{\bullet-}$ [46]. This disease is characterized by severe infections and a drastically decreased live expectancy. This demonstrates the important roles of macrophages and $O_2^{\bullet-}$ in the immunologic host defense. As a signaling molecule in inflammation $O_2^{\bullet-}$ enhances the release of pro-inflammatory cytokines such as tumor necrosis factor alpha (TNF-α) [25]. TNF-α may have proproliferative effects in hepatocytes thereby contributing to tumor promoting. Furthermore superoxide is metabolized to various reactive oxygen species (ROS), which may damage macromolecules in cells including DNA, thereby killing pathogens. In general a molecule with the capacity to damage macromolecules in pathogens might also damage structures in host cells as well. Under the assumption that $O_2^{\bullet-}$ is metabolized to reactive oxygen radicals that may damage DNA an enhanced $O_2^{\bullet-}$ generation during an inflammation might form the basis of inflammation-induced carcinogenesis [48].

$O_2^{\bullet-}$ produced in Kupffer cells might therefore not only be involved in mediating inflammation as a signaling molecule (cytokine) but might also be causative for DNA damage, thereby constituting an endogenous factor that is involved in the process of chemically-induced carcinogenesis.

2 Objectives

To provide accurate and reproducible quantification of DNA adducts at background and induced levels, appropriate analytical techniques are required. Therefore, the first part of this work was devoted to the development and validation of liquid chromatography tandem mass spectrometry (LC-MS/MS) methodology for the quantification of the DNA adducts 7-methylguanine (7-mG), O^6-methyl-2´-deoxyguanosine (O^6-mdGuo), 8-oxo-7,8-dihydro-2´-deoxyguanosine (8-oxodGuo), and 1,N^6-etheno-2´-deoxyadenosine (εdAdo), including the synthesis of adduct standards that are not commercially available and the internal standard O^6-[$^2H_3$]mdGuo.

DNA strand breakage as a consequence of direct interaction, e.g., by ionizing radiation, or of DNA adducts can be assessed by the comet assay (single-cell gel electrophoresis). However, DNA fragmentation also occurs during cell death and may confound the comet assay analysis as a quantitative endpoint of DNA damage. Therefore, the second aim of this work was to elucidate whether DNA fragmentation in the course of apoptosis might constitute a bias for the interpretation of the comet assay.

The biological significance of DNA adducts depend on the biochemical and biological consequences of different adducts, i.e., the probability of "primary DNA lesions" (DNA adducts) to be expressed as heritable genetic changes. In view of background DNA damage, the quantitative relationship between background adduct

levels and exposure-related increments are important elements of risk characterization. The question is to what extent increments in adducts result in increments in subsequent endpoints of genotoxicity and mutagenicity. The third aim of this work was therefore to investigate background DNA damage and dose-related increments *in vitro* using different genotoxicity endpoints. DNA adduct formation (methylation) was measured in L5178Y mouse lymphoma cells concurrently to the analysis of three standard genotoxicity endpoints, (i) comet assay, (ii) micronucleus test and (iii) L5178Y mouse lymphoma thymidine kinase mutation assay to determine DNA breakage, chromosomal damage and mutant frequency, respectively. The various quantitative endpoints were correlated with each other, including comparison of slopes at low effect.

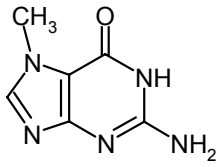
Changes in background DNA adduct levels may be observed *in vivo* also in the absence of a specific exposure to a DNA reactive agent, possibly mediated indirectly through complex host reactions that alter endogenous DNA adduct forming processes. Modulation of the production of reactive oxygen species may be particularly important in this context. This question was addressed in the fourth part of this work. It consisted of *in vivo* analyses of background oxidative DNA adducts in the liver of NADPH oxidase knock-out mice and their wild type counterparts. Tumorigenesis was initiated by a single dose of the methylating agent diethylnitrosamine followed by subsequent promotion by ethanol. Levels of 8-oxodGuo and εdAdo were determined to investigate whether $O_2^{\bullet-}$ produced in Kupffer cells may – besides being a cytokine – also influence background DNA damage, thereby constituting an endogenous factor possibly involved in the process of chemically-induced hepatocarcinogenesis.

3 Analytical method development

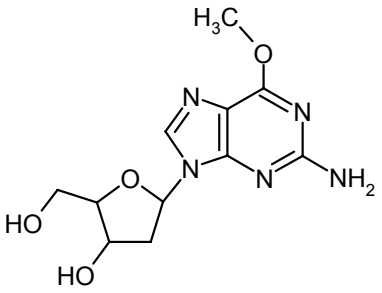
3.1 LC-MS/MS analysis of 7-mG, O^6-mdGuo, 8-oxodGuo, and εdAdo

To investigate the biological significance of DNA adducts, sensitive and specific analytical techniques are required to provide accurate and reproducible quantification. The adducts 7-mG, O^6-mdGuo, 8-oxodGuo, and εdAdo (Fig. 6) were chosen, since these adducts are formed both endogenously and exogenously by DNA-methylation, direct oxidation of DNA, and reaction of DNA with lipid peroxidation products. For all four adduct-specific methods have been described [49-53]. Application of most published methods is limited by the sample preparation necessary to quantify DNA adducts in samples isolated from tissue. This step is considered to be critical [35,54] because a complex and time-consuming off-line sample preparation can contribute to loss of analyte and, in the case of 8-oxodGuo, to the formation of artifacts by oxidation of dGuo [27]. On-line sample clean-up techniques coupled with mass spectrometry have proven to be efficient tools in sample preparation from biological matrices [53,55]. The effectiveness of column switching methods for the analysis of adducts was recently confirmed for DNA *in vitro* [33,56], nucleosides in urine [57] and for DNA in tissue [53,58,59].

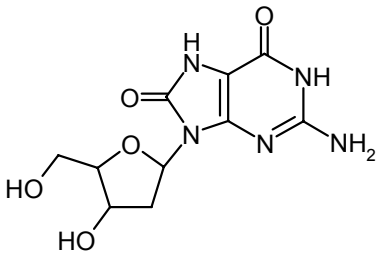
Therefore, a HPLC method using an on-line column switching technique was developed and validated for the simultaneous analysis of the promutagenic DNA adducts O^6-mdGuo, 8-oxodGuo, and εdAdo.



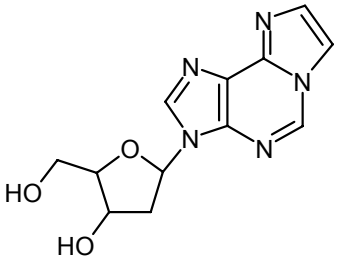
7-mG



O^6-mdGuo



8-oxodGuo



εdAdo

Fig. 6. Chemical structures of the DNA adducts 7-methylguanine (7-mG), O^6-methyl-2′-deoxyguanosine (O^6-mdGuo), 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxo-dGuo), and 1,N^6-etheno-2′-deoxyadenosine (εdAdo).

Since the nucleoside adduct 7-methyl-2′-deoxyguanosine is positively charged at the nitrogen in position 7, depurination occurs readily to yield 7-mG. Therefore, this type of methylation could not be analyzed as 2′-deoxynucleoside. A separate assay for the determination of the base adduct 7-mG was developed after thermal depurination from DNA prior to LC-MS/MS analysis.

3.2 Electrospray ionization

Electrospray ionisation (ESI) is a technique used to produce ionised analytes at atmospheric pressure [60]. The eluent of HPLC enters the electrospray source Fig. 7 and a fine spray of charged molecules in the presence of a strong electric field (2 to 5 kV) is generated. A flow of heated nitrogen gas is applied, resulting in solvent evaporation, leading to the formation of gasphase ions. The generated ions then pass through the orifice into the high vacuum region of the MS. The exact nature of the mechanism for the formation of charged ions from the spray is not clear. Theories have been proposed involving either coulombic fission, where large droplets divide into smaller droplets and eventually become single ions, or coulombic repulsion where ions are released from the droplet surface by overcoming the surface tension. ESI represents a soft ionization technique useful for polar biomolecules. For positive ionization acids such as formic acid can be added to the mobile phase to promote ionization by protonating e.g. amine groups of DNA bases.

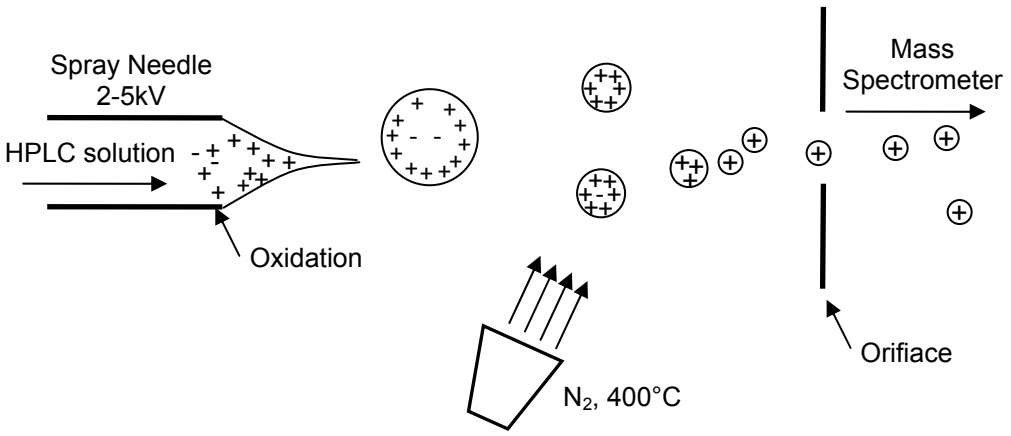


Fig. 7. Schematic representation of the positive electrospray ionisation process. Adapted from Cech and Enke [60].

3.3 Multiple reaction monitoring mode

The instrument used for DNA adduct analysis was a triple quadrupole tandem mass spectrometer run in the multiple reaction monitoring (MRM) mode. In this configuration the first (Q1) and third (Q3) quadrupoles are the mass analyzers (filters) (Fig. 8). Each consists of four parallel cylindrical rods to which voltages are applied to

generate oscillating electric fields. The second (Q2) quadrupole is the collision cell filled with an inert gas (nitrogen), in which analyte ions undergo collision induced fragmentation by application of collision energy [30].

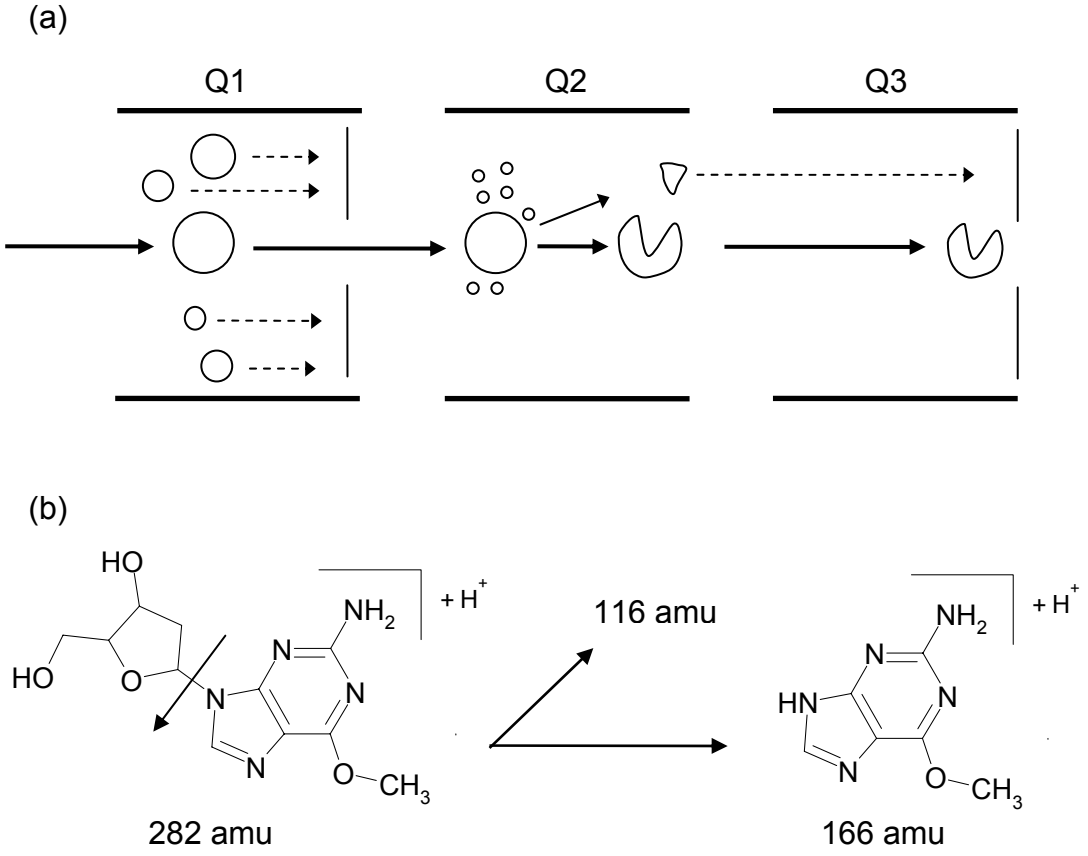


Fig. 8. (a) Schematic representation of the multiple reaction monitoring modus. The first quadrupole (Q1) filters the precursor ion that undergo collision with nitrogen gas in Q2 resulting in a specific fragment analyzed in Q3. (b) Example of MRM transition of a 2′-deoxynucleoside adducts O^6-mdGuo. The 2′-deoxyribose is lost as a neutral moiety (116 amu).

MRM involves the selection of a precursor ion of the compound of interest in Q1 which then undergoes fragmentation in the collision cell to a specific product ion, which is then detected by the third quadrupole (Q3). The mass analyzers Q1 and Q3 select the mass (m) to charge (z) ratio (m/z). If the charge is 1, then m/z is equal to the molecular weight of the ion or fragment under study. The 2′-deoxynucleosides selected in Q1 as $[M + H]^+$ undergo dissociation in the collision cell at relatively low collision energy, compared to the corresponding base or 2′-deoxynucleotide adducts [30] and all 2′-deoxyribonucleoside DNA adducts share a common fragmentation characteristic, which is the dissociation to the 2′-deoxyribose as a neutral moiety (116

amu) and the (adducted) base. This most prominent product ion formed $[B + H_2]^+$ could be analyzed in Q3.

Currently, the number of methods published for the determination of DNA adducts as 2′-deoxyribonucleosides by LC-ESI-MS/MS using MRM is increasing, since this method provides high specificity and sensitivity for quantification of DNA adducts. However, it can only be applied if the adduct is not too sensitive to depurination, as is the case with 7-methyl-2′-deoxyguanosine. In this situation the specific loss of 2′-deoxyribose could not be used and this adducts should be measured as the base adduct.

3.4 Experimental

3.4.1 Reagents

The adduct standards 7-mG, 8-oxodGuo and εdAdo as well as the chemicals for the synthesis of O^6-mdGuo and the internal standard O^6-[$^2H_3$]mdGuo were obtained from Sigma-Aldrich (Taufkirchen, Germany). Alkaline phosphatase from calf intestine was from Calbiochem (Darmstadt, Germany), Nuclease P1 was from MP Biomedicals, Inc. (Aurora, Ohio). HPLC-grade acetonitrile, methanol and water for liquid chromatography were purchased from Roth (Karlsruhe, Germany).

3.4.2 Synthesis of O^6-mdGuo and the labeled internal standard

O^6-mdGuo and its stable isotope-labeled analogue O^6-[$^2H_3$]mdGuo were synthesized based on a published method [61]. First sodium methoxide-solution was generated by adding peaces of sodium (310 mg) to 50 ml of cold CH_3OH (for the synthesis of O^6-mdGuo) or C[$^2H_3$]OH (for the synthesis of O^6-[$^2H_3$]mdGuo). Second 2′-deoxyguanosine (dGuo) (94 mg, 0.33 mmol) was dissolved in 1.7 ml of dry pyridine with 0.4 ml (2.8 mmol) trifluoroacetic anhydride on ice under dry nitrogen atmosphere. To this solution 50 ml of the respective methoxide solution was added drop wise on ice. The reaction was stopped after 48 h with pyridine hydrochloride (3 ml of pyridine/1 ml of HCl 30%). Then 0.4 g of $NaHCO_3$ was added. The solution was evaporated to dryness using a rotary evaporator. The residue was dissolved in 3 ml H_2O and injected in fractions of 100 µl into a preparative HPLC column

(Nucleosil® 100-7 C18, 250 mm x 10 mm, Machery-Nagel, Düren, Germany) using a gradient of 2 to 30% acetonitrile in water in 30 min at a flow rate of 2 ml/min and UV detection at 253 nm. The appropriate fractions were collected, combined and evaporated to dryness. O^6-[$^2H_3$]mdGuo and O^6-mdGuo were identified by MS/MS by recording enhanced product ion spectra on a QTrap (Applied Biosystems, Darmstadt, Germany). The standards were quantified by UV-spectroscopy (extinction coefficients ε(278 nm) = 8.51 cm^2/mmol and ε(274 nm) = 8.13 cm^2/mmol).

3.4.3 Liquid chromatography

For analysis of 7-mG base adduct 10 to 50 µl of sample were injected onto a reversed phase column (Reprosil Pur C18-AQ, 150 x 2.0 mm, 3 µm, Dr. Maisch, Ammerbuch, Germany). Solvent A was H_2O containing 3% acetonitril and 0.1% formic acid supplied isocratically with a flow rate of 240 µl/min for 5 min. Then the column was washed for 2 minutes with 100% solvent B (acetonitril). Prior to the next injection the column was equilibrated 10 min with solvent A.

For the analysis of O^6mdGuo, 8-oxodGuo and εdAdo column switching was performed. The configuration of the on-line extraction LC-MS/MS system is shown in Fig. 9. The center element of the system was an electrical valve (Valco Valve), which controlled the flow of the solvents from two pumps into two different columns. In the loading position (Fig. 9a) the autosampler (Agilent Series 1100, Waldbronn, Germany) introduced the sample into the system and pump 1 (Agilent Series 1100, Waldbronn, Germany) carried the aqueous mobile phase (5% methanol in 10 mM ammonium acetate, pH 4.3) at 1.5 ml/min to load the sample on the trap column (Oasis® HLB 25 µm 20 x 2.1 mm, Waters, Milford, Massachusetts). Sample loading and elimination of matrix components was complete after 1.5 min. Then the valve switched to the elution position (Fig. 9b). Pump 2 (Agilent Series 1100) supplied a gradient (8 min from 10 to 100% methanol) to back flush the trapped analytes from the trap column and to transfer them onto an analytical column (Reprosil ODS, C18, 3.5 µm, 150 x 4.6 mm, Dr. Maisch, Ammerbuch, Germany).

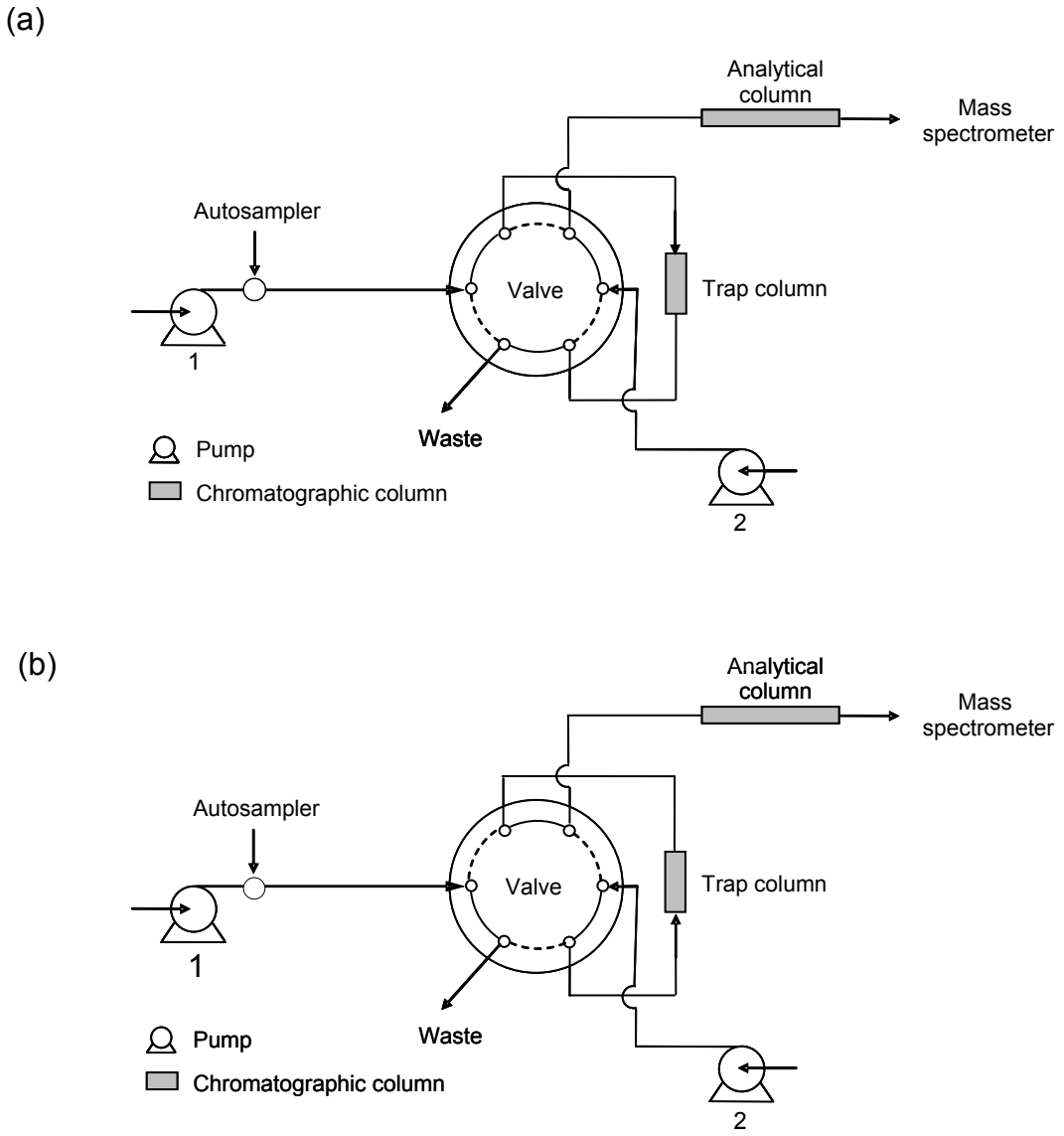


Fig. 9. Schematic configuration and valve positions of the on-line extraction LC-MS/MS system. (a) Sample loading position and (b) sample back flush and elution position.

3.4.4 Mass spectrometry

A triple-stage quadrupole mass spectrometer (API 3000, Applied Biosystems, Darmstadt, Germany) equipped with an Turbo Ionspray source was used. The ion spray voltage was 3500 V, the source temperature 400°C. The DNA adducts were monitored in the positive MRM mode. For optimum sensitivity, specific product ions and optimal instrument settings were obtained by infusion of authentic standards and using the quantitative optimization function of the Analyst 1.4.1 software (Applied Biosystems, Darmstadt, Germany). Table 1 provides the details of the instrumental settings. Two transitions per adduct were monitored. Peak areas of the most

sensitive transitions were used for quantification. For O^6-mdGuo, 8-oxodGuo and εdAdo standard curves were generated with O^6-[$^2H_3$]mdGuo as internal standard.

Table 1
Multiple reaction monitoring parameters for the analysis of the base adduct 7-methylguanine (7-mG) and the 2′-deoxyribonucleosides O^6-methyl-2′-deoxyguanosine (O^6-mdGuo), 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxodGuo), and 1,N^6-etheno-2′-deoxyadenosine (εdAdo) and the deuterated internal standard O^6-[$^2H_3$]mdGuo.

Analyte	Precursor ion (Q1)	Product ion (Q3)	DP	FP	CE	CXP
7-mG	166	149*	31	160	29	14
		124				12
O^6-mdGuo	282	166*	26	160	21	10
		149			47	12
8-oxodGuo	284	168*	26	130	19	14
		140			45	10
εdAdo	276	160*	31	180	23	14
		106			69	8
O^6-[$^2H_3$]mdGuo (IS)	285	169	26	160	21	10

*Transitions used for quantification
DP: declustering potential [V]; FP: focusing potential [V]; CE: collision energy [V]; CXP: collision cell exit potential [V].

3.4.5 Method validation

The two LC-MS/MS methods (one for 7-mG and the column switching method for O^6-mdGuo, 8-oxodGuo and εdAdo) were characterized for dynamic range, limit of detection (LOD: defined by a signal-to-noise ratio (S/N) ≥ 3), and limit of quantification (LOQ: S/N ≥ 7.5). The injection volume for 7-mG was 10 µl containing concentrations of the respective standard ranging from 0.1 to 30 pmol/ml. To compensate for matrix effects the validation of the column switching method was conducted in an artificial matrix consisting of the 4 normal 2′-deoxyribonucleosides (21% each dGuo and dCyd and 29% each dAdo and dThd), similar to the hydrolysis product of mammalian DNA. Each injection (100 µl) contained 320 nmol of blank matrix (equivalent to 100 µg DNA), the internal standard (1 pmol), and the respective working standards at 11 concentrations (spacing by factor 2) in the range from

0.12 pmol/ml to 125 pmol/ml. To assess inter-day precision and accuracy measurements were carried out with independently prepared standards on 5 different days.

3.4.6 Animals and administration of dimethylnitrosamine

All work conducted with animals was performed in compliance with the policies set forth in the Guide for the Care and Use of Laboratory Animals. Female Fischer F344 rats, 12 weeks old (about 200 g) were held on sawdust for 1 week for acclimatization. They were fed *ad libitum* with standard diet and had free access to tap water. The rats were dosed with dimethylnitrosamine (50 µg/kg b.wt.) in water by oral gavage after 6 h without access to food. After 60 min the rats were killed by asphyxiation with CO_2 and livers were removed. The livers were shock frozen in liquid nitrogen and stored at -80°C until analysis.

3.4.7 DNA isolation and DNA hydrolysis

DNA was isolated from rat liver using a DNA isolation kit (Nucleobond® AXG, Macherey-Nagel, Düren, Germany). Briefly, 250 mg of rat liver was homogenized mechanically (Ultra Turrax). The homogenate was treated with RNase A (200 µg/ml) and proteinase K (400 µg/ml). Then the lysate was transferred to a Nucleobond® AXG cartridge for adsorption of the DNA. After washing, elution, precipitation with isopropanol, and centrifugation a DNA pellet was obtained. A second elution step was carried out using the same volume of elution buffer. The DNA from the first elution/precipitation step (yield >100 µg of DNA) was used for the analysis of the 2′-deoxyribonucleosides O^6-mdGuo, 8-oxodGuo and εdAdo by column switching. The DNA from the second elution/precipitation step (yield ~ 20 to 40 µg of DNA) was used for the analysis of 7-mG. After dissolving the DNA pellets in water to a concentration of > 1 µg/µl, the concentration and purity were determined by UV spectroscopy by measuring the OD at 260 and 280 nm. Ratios of OD 260 to 280 between 1.8 and 2.0 were considered as clean. Prior to DNA hydrolysis the concentration was adjusted with water to 1 µg/µl.

To digest the DNA (yield of first elution step) to the 2′-deoxyribonucleosides, 2.5 µl of a NH_4Ac (1 mol/l, adjusted to pH 5.1 with acetic acid) buffer containing $ZnCl_2$ (45 mmol/l) was added per 100 µl sample. Internal standard (1 pmol) was added and

the sample was incubated with nuclease P1 (0.1 units per µg DNA) for 60 min at 40°C. Then 10 µl of a NH_4HCO_3 (1.5 mol/l, adjusted to pH 8 with ammonium hydroxide) buffer was added prior to incubation with alkaline phosphatase (0.02 units/µg DNA) for 60 min. To remove protein the sample was centrifuged in a 5000 NMWL cut-off filter tube (Ultrafree, Millipore, Eschborn, Germany) at 4°C and 5000 x g until the sample was completely filtered (30 - 60 min).

For neutral thermal depurination the DNA pellet of the second elution step was dissolved in 50 µl of H_2O. Then 90 µl of H_20 and 10 µl of NH_4HCO_3 (1 mol/l; pH 7.0) were added prior to incubation at 70°C. After 7 h the samples were transferred to a cut-off filter tube (Ultrafree 5000 NMWL, Millipore, Düren, Germany) and centrifuged at 4°C and 5000 x g until the sample was completely filtered (30 - 60 min).

3.5 Results and Discussion

3.5.1 Synthesis of O^6mdGuo and the internal standard

In Fig. 10 the proposed route for the synthesis of O^6-mdGuo and the internal standard O^6-[$^2H_3$]mdGuo *via* a putative 6-pyridyl intermediate is shown. For both O^6-mdGuo and O^6-[$^2H_3$]mdGuo the same experimental procedure could be used. The only difference was the generation of sodium methoxide-solution. CH_3OH, was used for the synthesis of O^6-mdGuo and C[$^2H_3$]OH for the synthesis of O^6-[$^2H_3$]mdGuo.

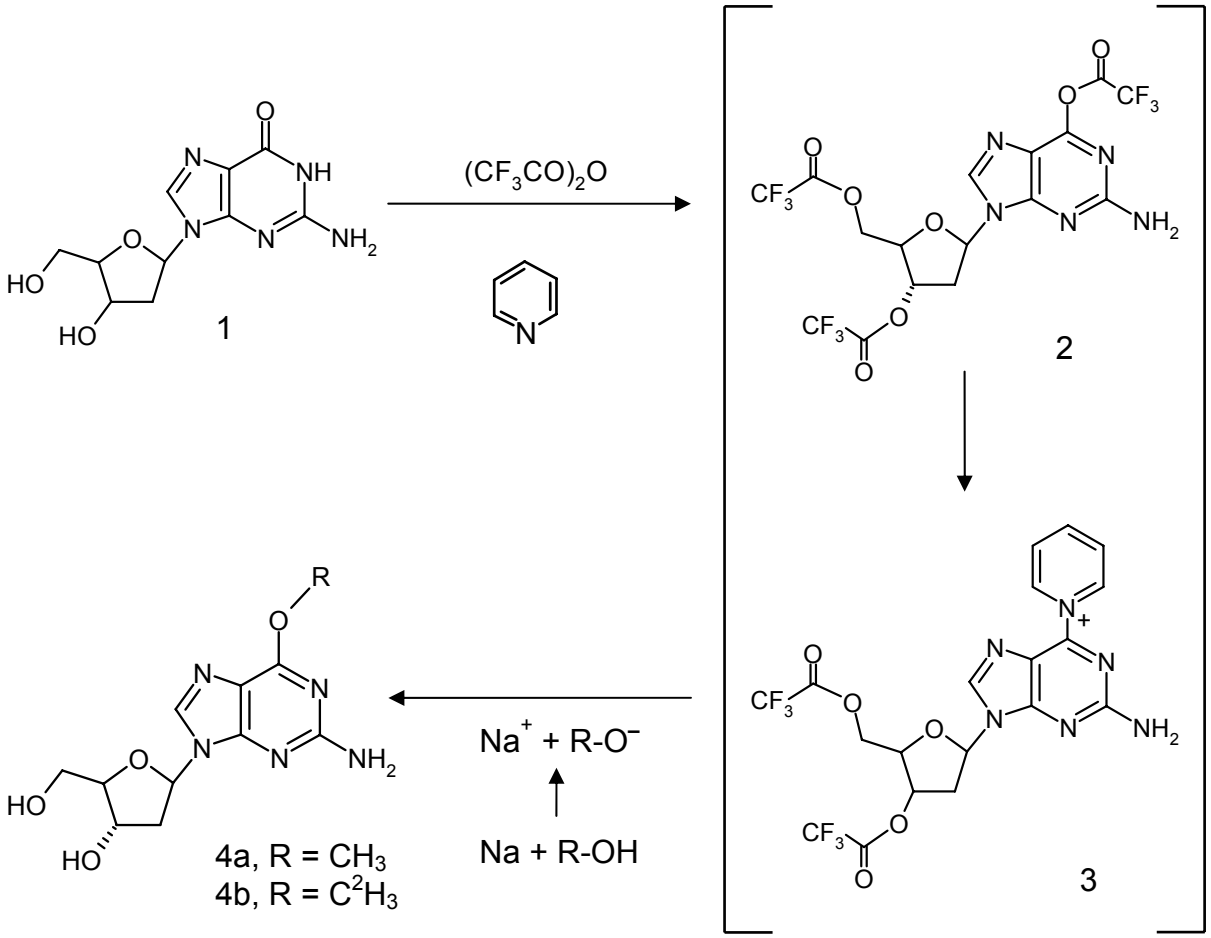


Fig. 10. Route for the synthesis of O^6mdGuo and the internal standard O^6-[$^2H_3$]mdGuo. Reaction of 2′-deoxyguanosine (1) under the described condition with trifluoractic anhydride in pyridine leads to the 6-trifluoracyl compound (2), where the trifluoracetyl group subsequently is displaced by pyridine to give (3). Conversion to O^6-mdGuo (4a) and O^6-[$^2H_3$]mdGuo (4b) involved the reaction with the corresponding sodium methoxide solution.

The synthesis was based on a protocol [61] published for the synthesis of 6-substituted 2′-deoxyguanosines derivatives. However, the description of the procedure gave only poor information on the reaction times, concentrations and temperatures. The trifluoracetic anhydride reaction was completed within 10 to 15 minutes on ice, but in preliminary experiments the addition of the quantity of sodium methoxide suggested by the authors resulted in our hands in a dark colored mixture in which the expected product was present only in very poor yields, not sufficient to work with low quantities of costly deuterated chemicals. The yield could be increased to approximately 60% by diluting the concentration of methoxide and adding the solution drop wise on ice over a period of 48 hours. The color of the solution had to be kept at a pale-yellow. If the solution turned slightly into red-brown the addition was stopped until the solution brightened again. The structural characterization of the DNA adduct standards by enhanced product ion spectra is shown in Fig. 11.

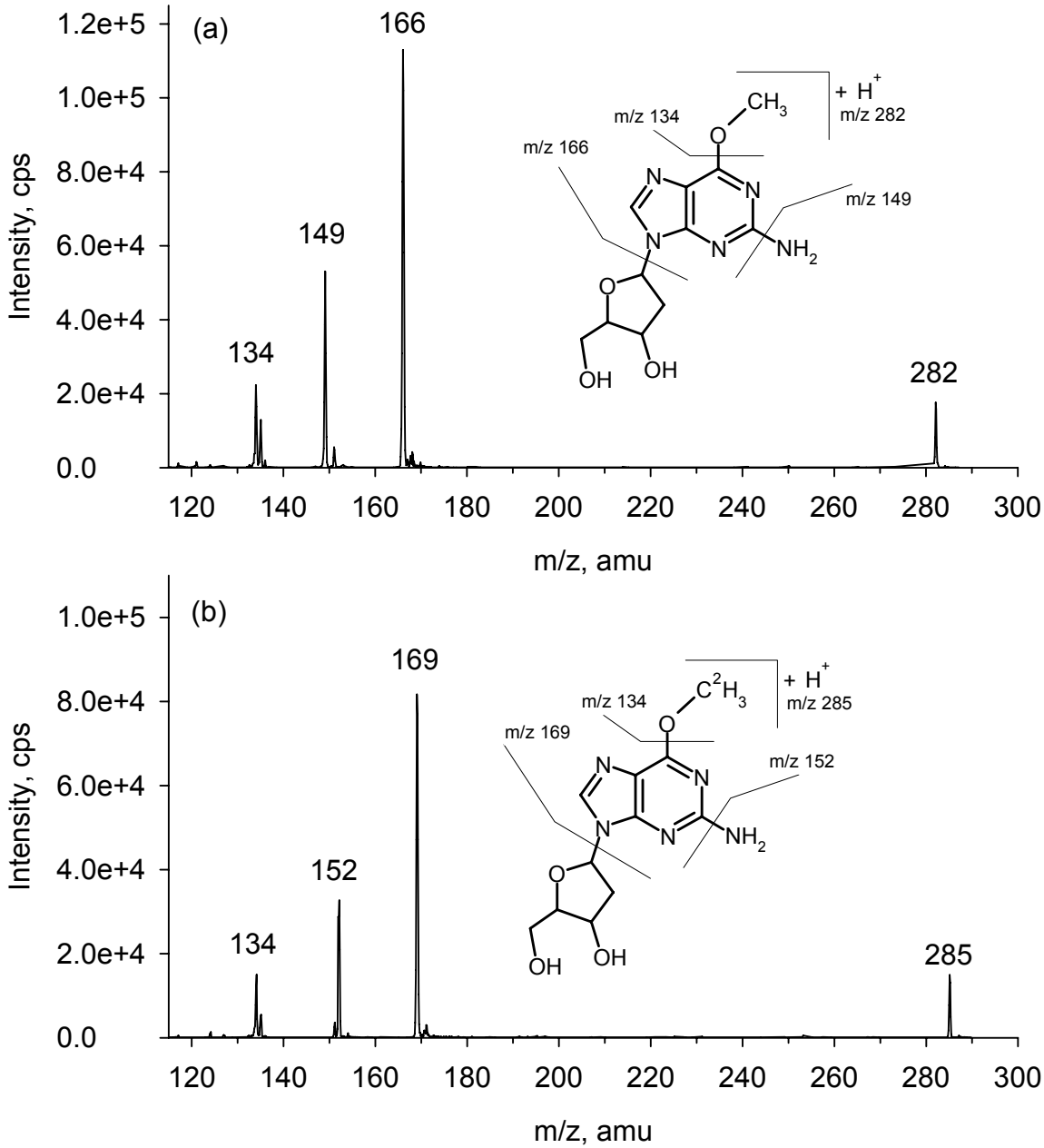


Fig. 11. Enhanced product ion spectra and proposed fragmentation of the synthesized DNA adduct standards (a) O^6-mdGuo and (b) O^6-[$^2H_3$]mdGuo. The precursor and main fragment ions of the internal standard O^6-[$^2H_3$]mdGuo are 3 amu greater.

3.5.2 Analysis of 7-mG

Methylation of 2′-deoxyguanosine in position 7(N) results in a formal positive charge which destabilizes the N-glycosidic bond [62]. Therefore, the nucleoside 7-methyl-2′-deoxyguanosine is unstable and readily depurinates [63]. It was shown that 7-methyl-2′-deoxyguanosine released from DNA rapidly decomposed at 37°C within a few

hours [50]. Therefore, the determination of the 2′-deoxynucleoside after enzymatic hydrolysis of DNA at 40°C would not accurately represent the total 7(N)-methylation of guanine in DNA. Consequently, a method was developed to measure the stable purine adduct. The purine 7-mG was released from DNA by neutral hydrolysis, since these conditions specifically result in depurination of 7-mG whereas the normal (not adducted) bases remain mainly at the DNA backbone [64]. They can be removed from the sample together with the phosphate backbone by filtration. The resulting filtrate could be injected into the HPLC-MS/MS system without any further work-up. The quantification of 7-mG was carried out using authentic 7-mG standard for calibration. No stable isotope labeled analogue was available for internal standardization. The validation results are listed in Table **2** and were within acceptable limits. Typical MRM chromatograms for the two transitions monitored for 7-mG are shown in Fig. 12.

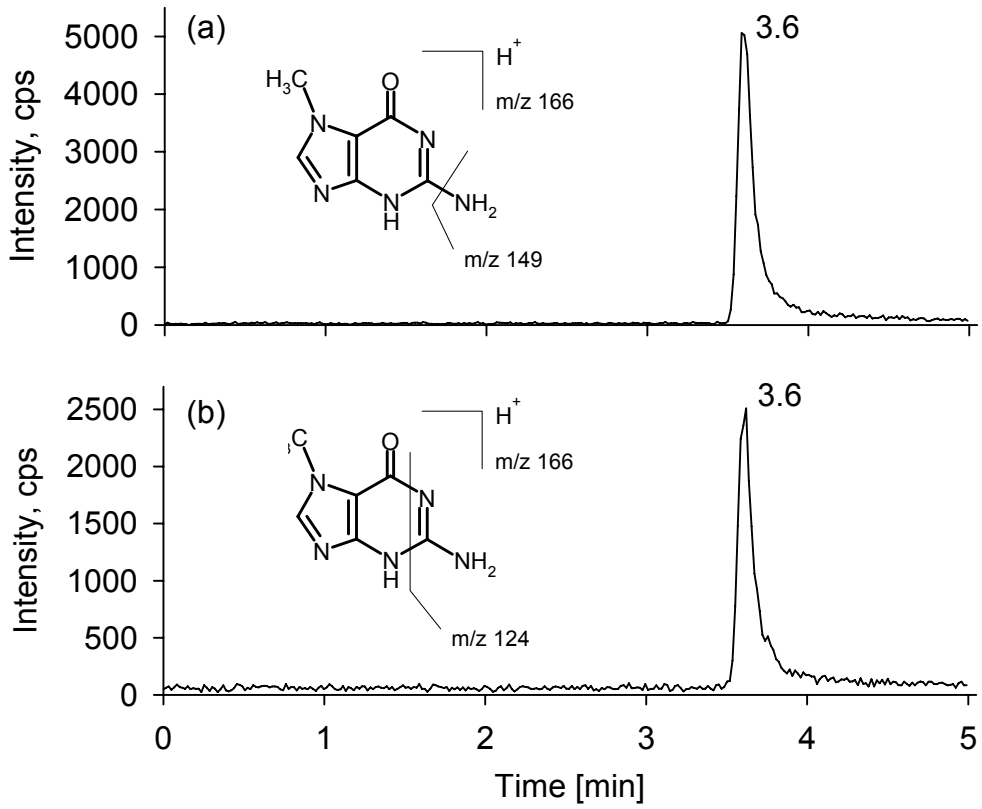


Fig. 12. Multiple reaction monitoring (MRM) chromatograms for 7-mG. Two transitions were monitored. (a) *m/z* 166 → 149 amu, and (b) *m/z* 166 → 124 amu. The inserts give structures and the m/z of the proposed MRM transitions.

3.5.3 Column switching LC-MS/MS method

Sample clean-up and chromatography of O^6-mdGuo, 8-oxodGuo and εdAdo were performed on-line by a column-switching set-up of two HPLC columns and included change in flow direction on the trap column (Fig. 9). It started with sample loading, trapping of the analytes on the trap column while simultaneously washing matrix components to the waste [55]. Due to similar molecular weights, polarities, and chromatographic properties of the DNA-adducts and the unmodified ("normal") nucleosides, it was necessary to optimize the time for loading/washing the trap column and the concentration of organic solvent in the mobile phase. Using 1.5 min for loading/washing and 5% methanol in the solvent, O^6-mdGuo, 8-oxodGuo, and εdAdo could be quantitatively and reproducibly retained on the trap column. After switching the valve, the compounds were back flushed with a higher organic solvent content onto the analytical column. Preliminary evaluation of the method showed that the normal nucleosides had not been completely washed from the trap column due to their high concentration. However, the reduction was sufficient to allow for adequate chromatographic peaks for all DNA-adducts (Fig. 13), as well as reproducible quantification. For the quantification of O^6-mdGuo the use of its labeled analogue O^6-[$^2H_3$]mdGuo as an internal standard was the most favorable choice. For 8-oxodGuo and εdAdo O^6-[$^2H_3$]mdGuo was able to compensate for variability during sample preparation and injection, but not for differences due to co-elution with other compounds. Since O^6-mdGuo exhibited the lowest background levels in biological samples [65] experimental parameters were optimized for this adduct, while keeping acceptable conditions for the quantification of 8-oxodGuo and εdAdo in the same analytical run.

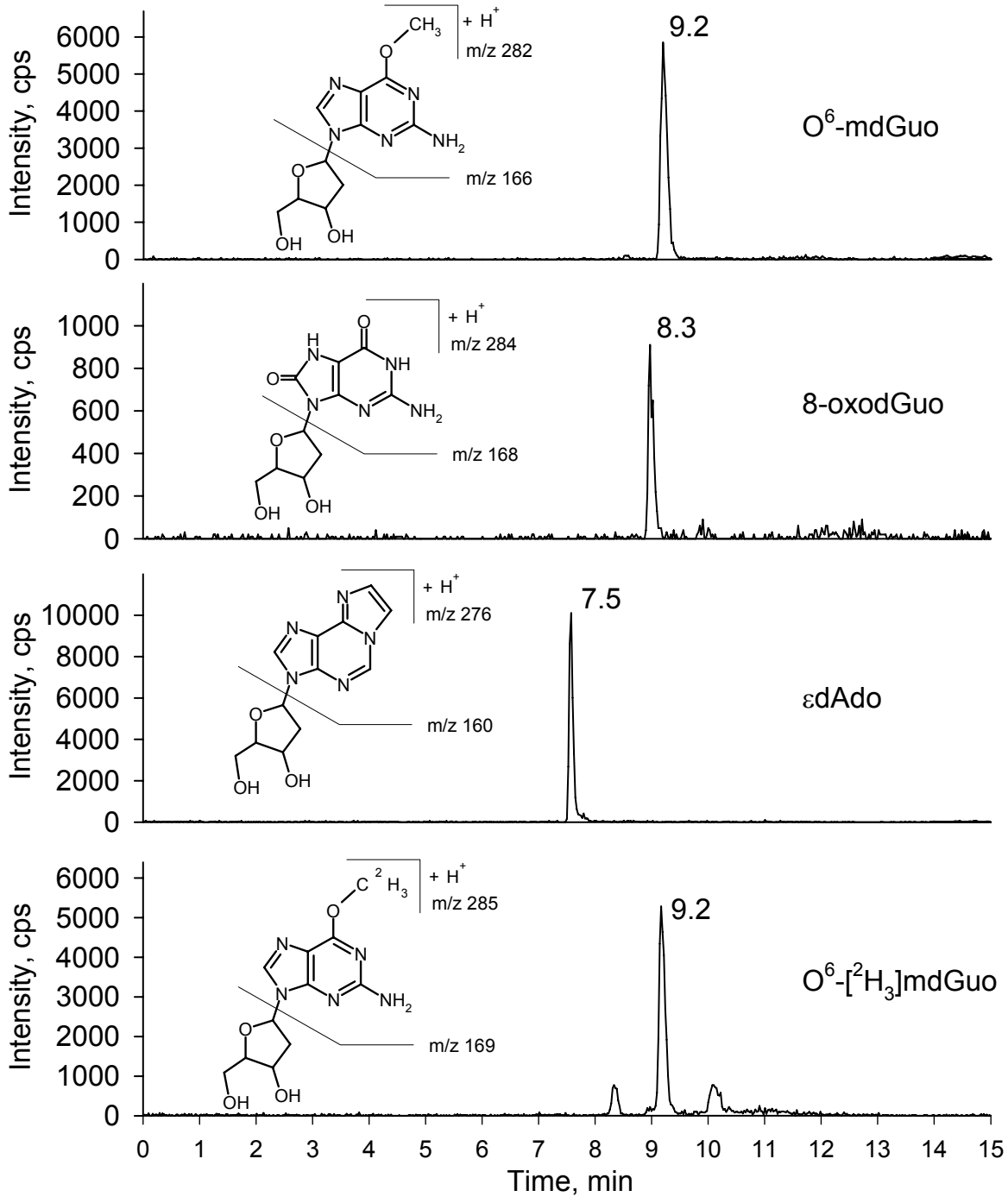


Fig. 13. Multiple reaction monitoring (MRM) chromatograms for the three analytes O^6-methyl-2′-deoxyguanosine (O^6-mdGuo), 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxodGuo), and 1,N^6-etheno-2′-deoxyadenosine (εdAdo) (all 8 pmol/ml), and the internal standard O^6-[$^2H_3$]mdGuo. The inserts give structures and the m/z of the respective MRM transitions.

3.5.4 Column switching: Method validation

Method validation was conducted with an injection volume of 100 µl containing 2′-deoxyribonucleosides as matrix, equivalent to a digest of 100 µg of DNA (320 nmol total nucleosides). The matrix was analyzed and contained no measurable levels of DNA adducts. Only upon storage of the matrix solution for more than 2 months in the refrigerator did oxidative reactions occur, which resulted in the formation of low amounts of about 100 fmol 8-oxodGuo.

The LOQs for O^6-mdGuo, 8-oxodGuo, and εdAdo are listed in Table 2. For a sample of 100 µg of DNA this corresponds to 75, 300 and 150 adducts per 10^9 normal nucleosides, respectively. The LOQ for O^6-mdGuo was about 4-fold better compared to a previously published LC-MS/MS method [49]. The sensitivity of our method for εdAdo was comparable to other reports [51]. Since the method here was optimized for O^6-mdGuo, the settings were not the best possible for 8-oxodGuo. Therefore, the LOQ for 8-oxodGuo was about 2 to 5 fold higher compared to published LC-MS/MS methods [50,65]. However our LOQ was satisfactory because the lowest value published for 8-oxodGuo in DNA from rat liver is approximately 500 adducts per 10^9 nucleosides [18,66], thus about twice our LOQ.

Table 2 summarizes the assay characteristics for the adducts monitored on 5 different days. The matrix-matched calibration curves were linear for O^6-mdGuo, 8-oxodGuo, and εdAdo in the range of 0.24 to 125 pmol/ml, 0.98 to 125 pmol/ml, and 0.49 to 62.5 pmol/ml, respectively. Within the dynamic range linear correlation coefficients *r* were better than 0.98 for all analytes. The inter-day precision varied in the range of 1.7 to 9.3%, 10.6 to 28.7% and 6.2 to 10.4% for O^6-mdGuo, 8-oxodGuo, and εdAdo, respectively. The inter-day precision of 8-oxodGuo was not as good as for the two other adducts. The formation of 8-oxodGuo from dGuo requires only one oxidation step and the artifact formation of 8-oxodGuo has been discussed extensively in the literature [66-68]. In general published 8-oxodGuo levels have to be seen critically since the formation of 8-oxodGuo in the presence of large concentrations of dGuo is difficult to control [69]. In comparison to other methods based on LC-MS/MS our method appeared to produce low levels of artifact 8-oxodGuo probably due to reduced sample work-up by on-line sample preparation using the column switching unit.

Table 2
Analytical performance for the determination of 7-methylguanine (7-mG), O^6-methyl-2′-deoxyguanosine (O^6-mdGuo), 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxodGuo), and 1,N^6-etheno-2′-deoxyadenosine (εdAdo). The validation parameters, limit of detection (LOD: defined by a signal-to-noise ratio (S/N) ≥ 3), and limit of quantification (LOQ: S/N ≥ 7.5) listed as fmol on column (o.c.) and as adducts per 10^9 normal nucleotides (nt), dynamic range, accuracy, inter-day precision (at 10-fold LOQ) were determined in 5 independent experiments on 5 different days. All concentrations in pmol/ml.

Parameter	7-mG	O^6-mdGuo	8-oxodGuo	εdAdo
LOD [fmol] o.c.	10	12	49	24
LOQ [fmol] o.c.	30	24	98	48
Dynamic range	0.3 - 30	0.24 - 125	0.98 - 125	0.49 - 62.5
Accuracy	89 - 110%	94 - 105%	85 - 119%	85 - 108%
Inter-day precision (10 x LOQ)	5.7 %	5.2%	18.2%	6.2%
Regression factor	0.997	0.991	0.98	0.989

3.5.5 Analysis of adducts in rat liver

To demonstrate applicability of the method to DNA isolated from rat liver, the validated methods were applied to liver samples from female F344 rats. The LC-MS/MS analysis of 7-mG conducted after neutral thermal depurination is shown in Fig. 14. Forty µg of DNA were sufficient for the detection of background levels and was determined as 850 per 10^9 normal nucleotides for the sample shown Fig. 14. Due to its relative high abundance and ease of measurement as a DNA lesion 7-mG was used as a biomarker [71,72]. However, since only weakly promutagenic it is considered to be of minor biological relevance for tumorigenesis. Literature values for background levels in rat liver vary significantly. For example 260 7-mG per 10^9 normal nucleotides (0.91 pmol 7-mG per µmol dGuo) were determined by GC-MS [71] and 10,000 per 10^9 were measured by Park and Ames [73] by HPLC-ECD (electrochemical detector). However, such high values are questionable since this would mean that the level of 7-mG alone would be equal to the sum of all background DNA lesions estimated to be on the order of 10,000 per 10^9 [18]. The background levels for 7-mG determined here were on the same order of magnitude as the lowest level published for 7-mG so far. RNA contaminations have to be strictly

avoided, since the 5′-caps of mRNAs usually include a guanine methylated in 7(N) position. When analyzed as the purine adduct it can not be discriminated between 7-mG from DNA or mRNA. Therefore, in the assay here a RNase step was included in the DNA isolation procedure. 7-mG originating from mRNA contaminations might be the underlying cause for some extraordinary high literature values.

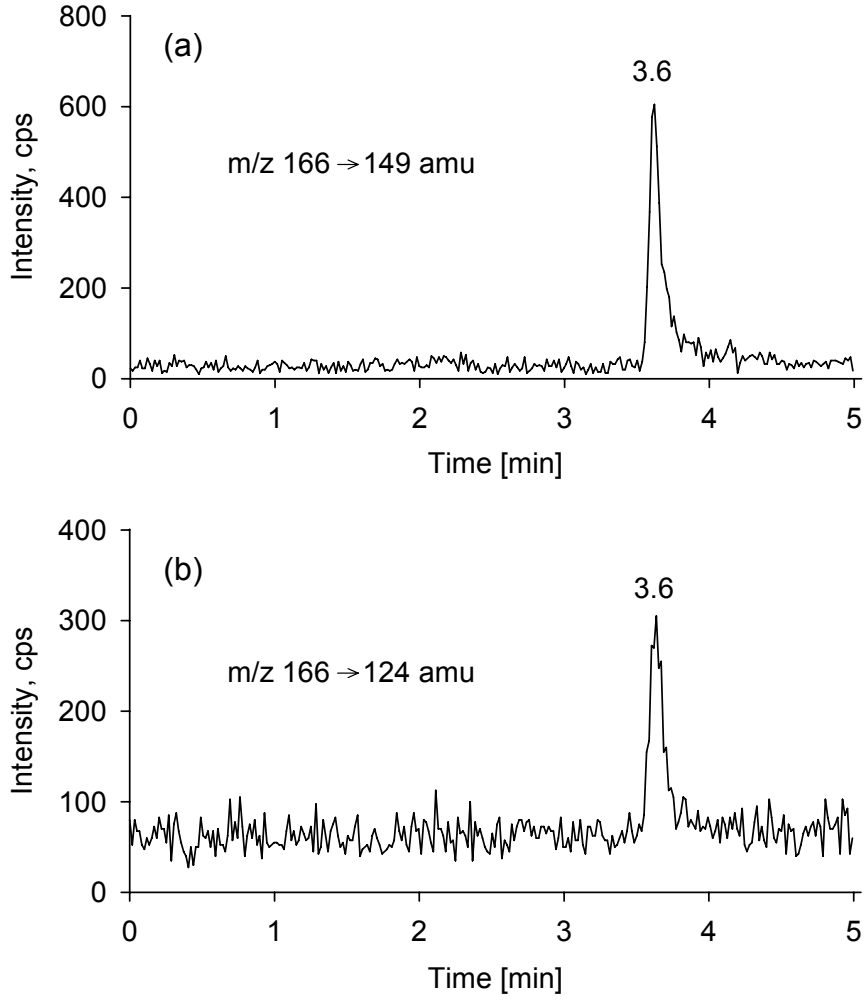


Fig. 14. LC-MS/MS analysis of 7-methylguanine (7-mG) in DNA isolated from liver of an untreated rat. Two transitions were monitored. (a) *m/z* 166 → 149 amu, and (b) *m/z* 166 → 124 amu.

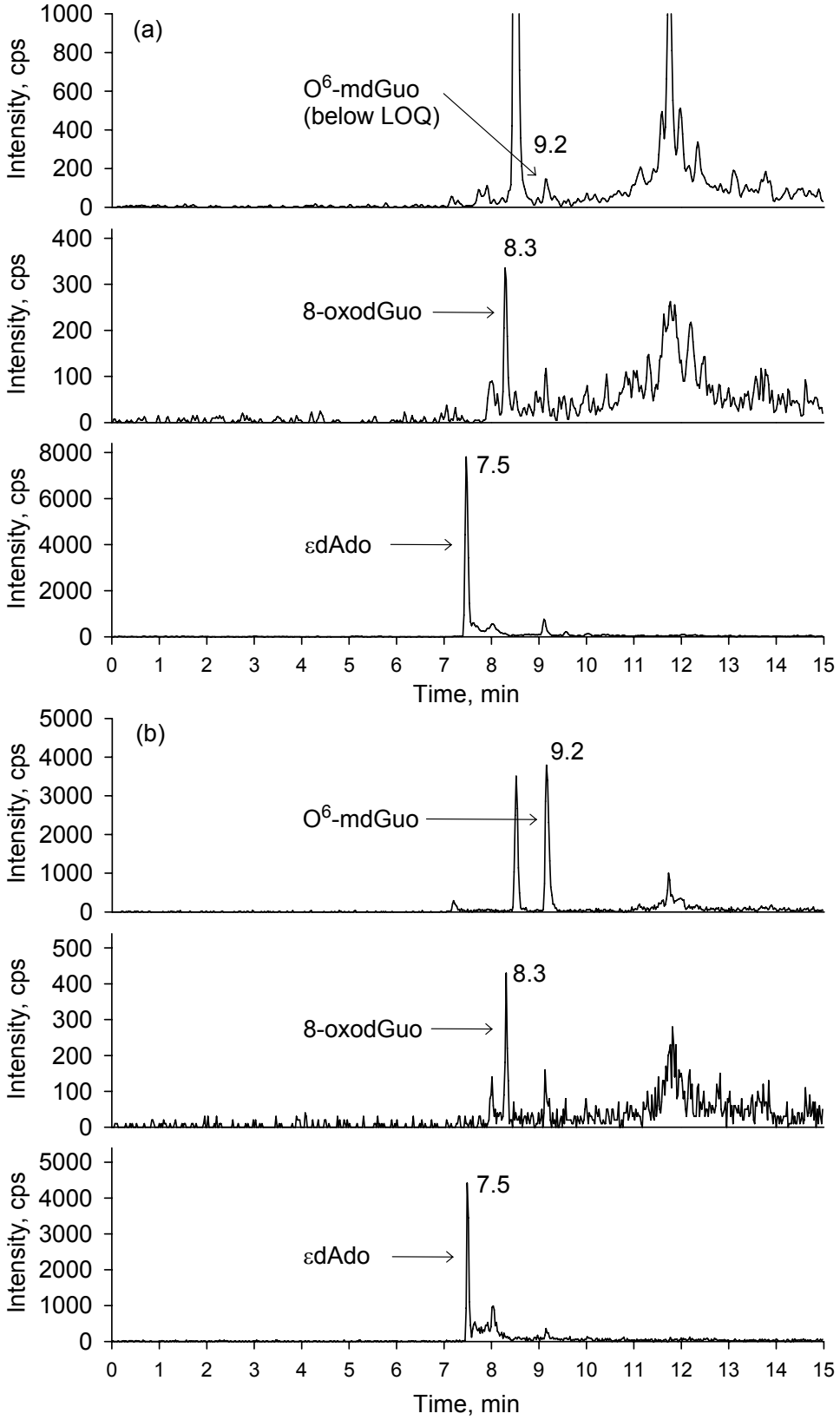


Fig. 15. LC-MS/MS analysis of O^6-methyl-2′-deoxyguanosine (O^6-mdGuo), 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxodGuo), and 1,N^6-etheno-2′-deoxyadenosine (εdAdo) in DNA isolated from liver of an (a) untreated rat and (b) of a rat one hour after treatment with 50 μg/kg b.wt. dimethylnitrosamine.

In samples from control rat liver O^6-mdGuo was below the LOQ but above the LOD of 37 adducts per 10^9 normal nucleosides. Literature values range from 0.3 adducts per 10^9 normal nucleosides determined by immunochemical analysis with a monoclonal antibody [74] and 1300 O^6-mdGuo per 10^9 normal nucleosides determined by a LC-MS/MS method [50]. For 8-oxodGuo we measured a background level of 520 adducts per 10^9 normal nucleosides. A large database on 8-oxodGuo exists and levels determined in liver of untreated rats show big variability [69]. The lowest published values for 8-oxodGuo were on the order of 500 adducts per 10^9 nucleosides [18,67], which corresponds to the level observed here. For εdAdo, we found 130 adducts per 10^9 nucleosides. This value is close to the level of 90 adducts per 10^9 nucleosides measured previously with LC-MS/MS in livers of untreated female Sprague-Dawley (SD) rats [52]. Besides the indicated background DNA lesions O^6-mdGuo, 8-oxodGuo, and εdAdo additional peaks appeared in the DNA adduct profile from rat liver (Fig. 15) when compared to the reference profile (Fig. 13). The only structural information that we can provide is the loss of 116 amu given by the neutral loss of 2-deoxyribose. It was assumed that these compounds are DNA adducts of other background origin that were retained on the trap column under the trapping conditions and were backflushed onto the analytical column together with the monitored adducts.

To verify the applicability of the method for the determination of O^6-mdGuo, two rats were treated by gavage with a single low dose of dimethylnitrosamine (50 µg/kg b.wt.). The levels of 8-oxodGuo and εdAdo, i.e., the two adducts associated with oxidative stress, remained unchanged. For O^6-mdGuo, a clear increase above background was seen, with a mean of 200 adducts per 10^9 normal nucleosides. (Fig. 15, bottom panels) shows the respective chromatograms. One h after oral gavage of female SD rats with 50 µg [^{3}H]DMNA per kg body weight, the level of tritiated O^6-mdGuo in liver had been reported to be about 0.3 µmol/mol dGuo [75]. This is equivalent to about 60 adducts per 10^9 normal nucleosides measured as an incremental DNA damage. Our value of 200 adducts per 10^9 nucleosides, which represents the sum of background and dose-related increment, fits to the published data.

In conclusion, new LC-MS/MS methods were developed and validated for the determination of 7-mG (as base adduct), O^6-mdGuo, 8-oxodGuo and εdAdo (as 2′-deoxyribonucleosides). The methods showed satisfactory sensitivity, precision and

accuracy to determine background (except O^6-mdGuo, between LOD and LOQ) and induced levels. These methods may be useful tools in studies evaluating the relationship between DNA adduct formation (for the adducts chosen) as an increment to background and biological response from e.g. alkylating agents.

4 Comet assay and cytotoxicity

4.1 The comet assay

The comet assay (single cell gel electrophoresis) is a fast and easy method to detect chemically-induced DNA damage, in particular DNA single and double strand breaks and alkali-labile sites. It is based on the migration of DNA fragments out of a cell nucleus into a surrounding agarose-gel during electrophoresis. With increasing numbers of DNA strand breaks, the amount of DNA fragments that migrate into the gel increases, forming "comet like" figures with "head" and "tail" observable under the microscope (Fig. 16).

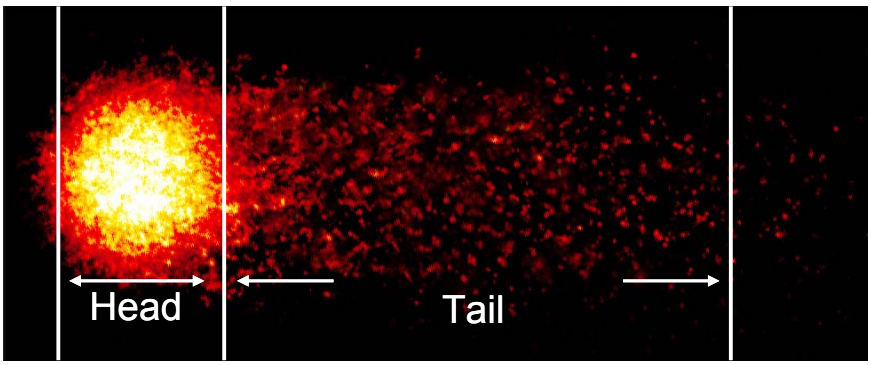


Fig. 16. Image of a damaged cell in the comet assay stained with propidium iodide. A computer-aided analysis system determines the extent of migrated DNA.

During the last 15 years the assay has emerged as a standard method for *in vitro* genotoxicity testing of chemicals and for the performance of the assay, guidelines and recommendations were published [76,77].

DNA migration in the comet assay allows no backtracking to the initial DNA damaging event. Moreover, DNA strand breaks might be caused by other than genotoxic effects. DNA fragmentation in the course of an apoptotic process may also give rise to DNA fragments that would migrate into the gel during electrophoresis and therefore may constitute a bias for the correct interpretation of the comet assay as a genotoxicity indicator [78].The distinction between genotoxic or non-genotoxic mode of action of carcinogens is crucial for comprehensive risk assessment of chemicals since it determines the model used for extrapolation from high to low dose within risk assessment. Comets with small or non-existent heads and the majority of DNA measured in the tail ("ghost cells") are generally interpreted as the microscopic images of apoptotic or necrotic cells [79]. Exclusion of these ghost cells from analysis and concurrent assessment of cell viability was recommended by an expert panel [77] for the correct interpretation of comet assay data in genotoxicity analyses. Still, it is not clear whether increased DNA fragmentation due to earlier stages of apoptosis can result in the generation of false positive results in the comet assay [76] even if the ghost cells are excluded.

4.2 Comet assay and sodium arsenite

Sodium arsenite ($NaAsO_2$) is classified by the International Agency for Research on Cancer (IARC) as a *human carcinogen* [80]. The underlying mechanisms have been subject of extensive research [81] but could still not be elucidated completely. For carcinogenic agents with unknown mode of action the comet assay may be used as a fast and easy method to indicate genotoxicity. In fact various studies employing the alkaline version of the comet assay indicated DNA damaging properties of $NaAsO_2$ *in vitro* [82-85]. Observations of increased DNA migration following $NaAsO_2$ treatment were interpreted as genotoxic DNA damage, indirectly mediated by reactive oxygen species induced by $NaAsO_2$. In contrast, $NaAsO_2$ revealed very low mutagenicity in various other in vitro test systems [86], while effectively triggering apoptosis in numerous cell lines and types [87].

As recently demonstrated [88] time factors are decisive for an accurate determination of $NaAsO_2$-induced cell death. More precisely, if the duration between the $NaAsO_2$ treatment and the assessment of cell viability by vital dye assays (based on plasma membrane integrity) was too short, the proportion of cells undergoing

apoptosis was drastically underestimated. These vital dyes that are often used to asses cell viability in parallel to the comet assay, enter the cells through damaged plasma membranes and are for that reason not indicative for apoptotic cells in early stages that still maintain plasma membrane integrity. The published comet assay studies applied a wide range of $NaAsO_2$ concentrations. However, in most studies the data were collected only at a single time point in the range of 0.5 to 8 hours [82-85].

The purpose of the following experiments was to determine whether $NaAsO_2$-induced effects in the comet assay could be explained on the basis of an induction of apoptosis and are therefore falsely interpreted as genotoxic DNA damage, even if the ghost cells are excluded from the analysis. We evaluated frequency histograms for single cell measures of % Tail DNA (% of DNA in comet tail) in 30-minute intervals after adding $NaAsO_2$ to L5178Y mouse lymphoma cells and defined limits of % Tail DNA to categorize cells as C0 (≤ 5%), C1 (5 < % Tail DNA ≤ 60%), and C2 cells (> 60%), indicative of "no damage", "intermediate damage", and "ghost cell", respectively. As positive controls we used dexamethasone as a non-genotoxic inducer of apoptosis [89,90] and the DNA-alkylating agent methyl methansulfonate as a genotoxic agent. Concurrently to the analysis of comets the percentage of annexin V-positive cells and propidium iodide-positive cells were evaluated by flow cytometry. Additionally, apoptotic cells were identified by the characteristic morphological changes using Hoechst bisbenzimide 33258 staining.

4.3 Experimental

4.3.1 Chemicals and reagents

Bisbenzimide H 33258 trihydrochloride (Hoechst 33258; CASNR 23491-52-3), dexamethasone (CASNR 50-02-2), horse serum, low melting agarose (CASNR 9012-36-6), methyl methansulfonate (CASNR 66-27-3), N-lauroylsarcosine sodium (CASNR 137-16-6), normal melting agarose, RPMI 1640, dimethyl sulfate (CASNR 67-68-5) L-glutamine (CASNR 56-85-9), penicillin, propidium iodide (CASNR 25535-16-4), sodium arsenite (CASNR 7784-46-5), sodium pyruvate (CASNR 113-24-6), sodium chloride (CASNR 7647-14-5), streptomycin, were purchased from Sigma Aldrich (Germany). Anti-bleaching solution for microscopic analyses was obtained

from Linaris (Germany). Fluorescein-conjugated annexin V (annexin V-Fluos) was purchased from Roche Diagnostics GmbH (Mannheim Germany).

4.3.2 Cell culture

L5178Y mouse lymphoma cells were routinely grown at 37°C in a 5% CO_2 atmosphere. Every 2 to 3 days the culture was diluted with fresh RMPI 1640 medium containing 10% horse serum, 2 mM L-glutamine, 20 mg/ml sodium pyruvate and antibiotics (penicillin, streptomycin).

4.3.3 Treatment of cells

Two hours prior to treatment L5178Y mouse lymphoma cells of an exponentially growing culture were seeded (200.000 cells/ml) in 100 ml medium. Then 100 µM $NaAsO_2$ (dissolved in H_2O), 100 µM dexamethasone (dissolved in DMSO) or 200 µM MMS (dissolved in DMSO) was added (final concentration of solvent 0.05%). The first sample was taken immediately after adding the substances, and then the flasks were kept at 37°C and in a 5% CO_2 atmosphere throughout the experiment. Every 30 min (60 min with dexamethasone) a sample (5 ml cell suspension) was taken and split to two portions each containing approximately 0.5 x 10^6 cells (2.5 ml). One fraction was prepared for analyses with the comet assay and Hoechst staining, the other was used for flow cytometry analysis.

4.3.4 Comet assay

The comet assay was performed according to recommendations published elsewhere [77]. Briefly, 20 µl of the cell suspension (≈1 mio cells/ml) were mixed with 180 µl of 0.5% LMP agarose and added to fully frosted slides that had been covered with a bottom layer of 1% HMP agarose. The slides were collected at 4°C in a cuvette containing lysis solution (2.5 M NaCl, 0.1 M EDTA, 0.01 M Tris and 1% Triton X-100, 10 g/l N-lauroylsarcosine sodium, to pH 10 with NaOH). The remaining fraction of cells was immediately used for Hoechst staining. All slides were placed in the electrophoresis solution (300 mM NaOH, 1 mM EDTA pH >13.0) for 20 min, and then electrophoresis was carried out for 20 min at 25 V (1.1 V/cm) and 300 mA. The slides were neutralized in 0.4 M Tris buffer (to pH 7.5 with HCl) and stained with 15 µl

propidium iodide solution (20 µg/ml). Images of 50 randomly selected cells (25 per replicate slide) for each sample were analyzed with a fluorescence microscope (Labophot 2, Nikon, Germany) using an image analysis software (Komet 5, BFI Optilas, Germany). The percentage of DNA in the tail was used to quantify DNA migration.

4.3.5 Apoptosis and cell viability

To analyse apoptosis microscopically by morphological features, approximately 30.000 cells per sample (from the remaining portion of comet assay preparation) were spun on slides (Cytospin 3, SHANDON, Germany) fixed with methanol (-20°C) and stained with Hoechst 33258 (50 µM, pH 7.0) for 5 minutes. The slides were washed three times with PBS (pH 7.0) and mounted with anti-bleaching solution (Linaris, Germany) prior to analysis. At every time point 2 replicate slides were prepared and 1000 cells were analysed per slide. Simultaneously to the comet assay preparation annexin V positive and propidium iodide positive cells were analysed by flow cytometry. Approximately 0.5 x 10^6 cells were immersed in 100 µl of binding buffer (BD, Heidelberg) that contained 0.5 µg/ml annexin V-Fluos and 20 µg/µl propidium iodide. After 15 min incubation at room temperature 400 µl of binding buffer were added before analysis. The samples were analysed with a flow cytometer from Becton Dickinson (BD–LSR) using an argon-ion laser (excitation at 488 nm, bandpass filter: 530 nm for annexin V-fluorescence and 670 nm bandpass filter for propidium iodide fluorescence). The fluorescence of 20,000 cells was acquired and analyzed with CellQuest Pro software (Becton Dickinson, Sunnyvale, USA). The events were gated to quadrants (Fig. 20). Annexin V-positive cells (lower right) were considered as early apoptotic cells, whereas propidium iodide uptake indicated necrosis (upper left) or necrosis and late apoptosis (upper right). Viable cells (lower left) are negative for both stains.

4.3.6 Analysis of comet assay data

For qualitative examinations the cells were classified into three categories. (i) C0-cells: % Tail DNA ≤ 5% no damage, (ii) C1-cells: 5 < % Tail DNA ≤ 60% intermediate damage, (iii) C2-cells: %Tail DNA > 60 % cells with small or non-existent heads and large diffuse tails (cells with cloud or ghost cell appearance) that would have been

excluded in genotoxicity analyses according to recommendations from the literature [77]. Based on our experience with $NaAsO_2$ all cells with %Tail DNA > 60 % displayed the microscopically appearance of ghost cells. The values of the C1 and C2 cells were documented in frequency histograms for %Tail DNA to illustrate the distribution of the single cells.

4.4 Results

4.4.1 Comet assay

Fig. 17 shows frequency histograms for %Tail DNA (from 5% to 100% Tail DNA, 5% Intervals) measured at different times after treatment of mouse lymphoma L5178Y cells with $NaAsO_2$. At time zero, all cells were in the category C0 (≤ 5%; data not shown). A progression to higher % Tail DNA with time was observed. At t = 60 minutes the number of cells with moderate DNA migration was increased, which included one cell of category C2. At t = 120 more C2 cells appeared. Only few cells showed up in the intermediate range of DNA migration. The resulting bimodal distribution with C0 + C1 cells on the left side and C2 cells on the right was even more pronounced at later time points. C0-cells were observable at all times (see inserts).

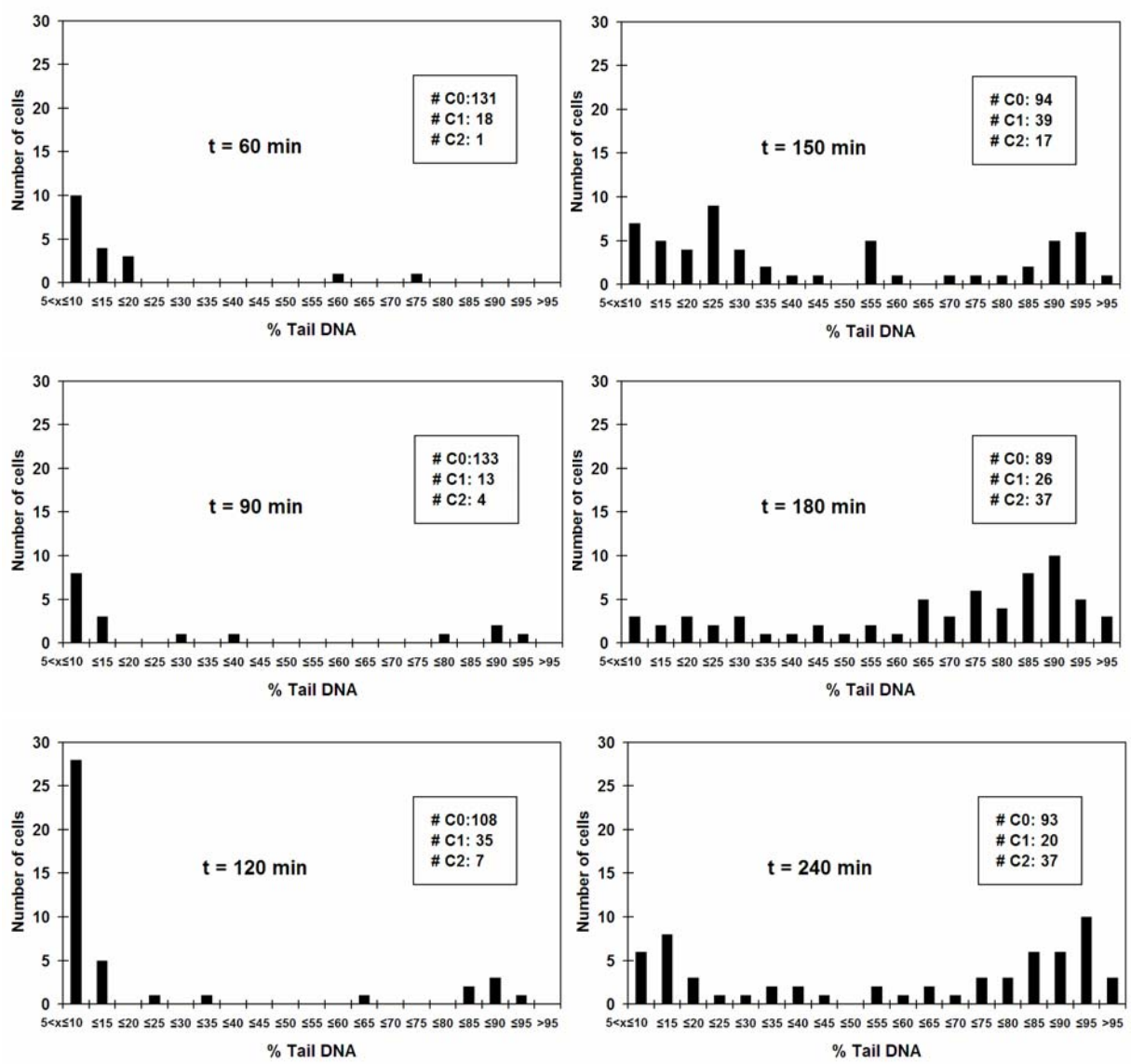


Fig. 17. Distribution of the percentages of migrated DNA in the comet assay of 150 cells pooled from three independent experiments, monitored at selected times after incubation of mouse lymphoma L5178Y cells in medium containing 100 µM $NaAsO_2$. The inserts give absolute numbers of cells in the following three categories: C0-cells: Tail DNA ≤ 5% (no damage; not shown in histogram), C1-cells: 5 < % Tail DNA ≥ 60% (intermediate damage) and C2-cells: %Tail DNA > 60 % (cells with small or non-existent heads and large diffuse tails).

The shift from category C1 to C2 is illustrated in Fig. 18, showing a peak of C1 cells at t = 150, followed by a maximum for C2 cells at later times (t = 180 to 240 minutes).

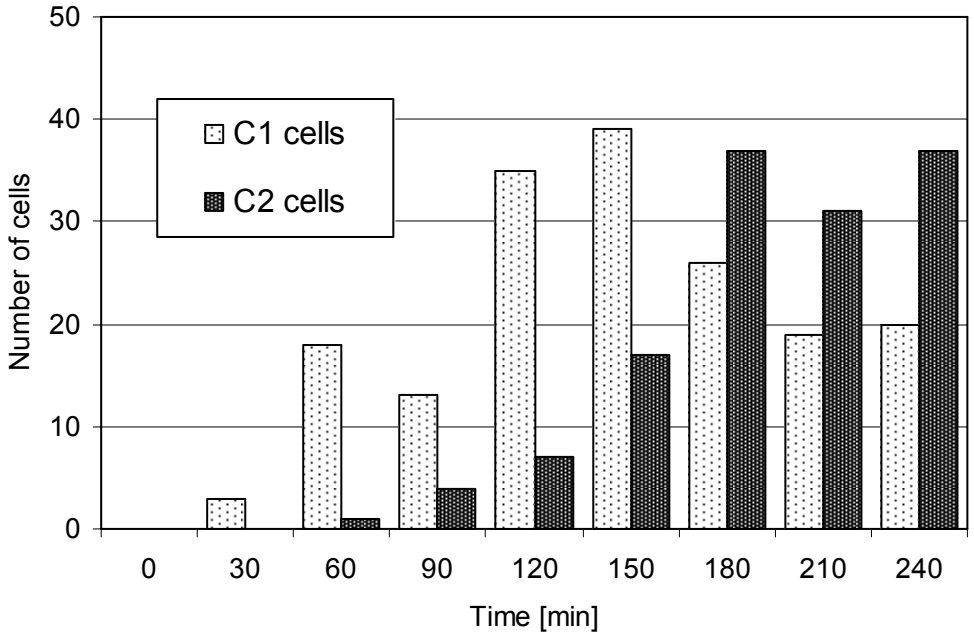


Fig. 18. Number of C1 and C2 mouse lymphoma cells obtained at different times after incubation in medium containing 100 µM $NaAsO_2$. C1-cells: 5 < % Tail DNA ≤ 60% intermediate damage, C2-cells: %Tail DNA > 60 %. See legend to Fig. 17 for characteristics of classification.

In order to compare the late-stage frequency histogram observed after $NaAsO_2$ treatment with chemicals that are generally accepted as non-genotoxic inducers of apoptosis or clearly genotoxic chemicals dexamethasone and MMS were used as positive controls. Fig. 19 compares the comet assay results. $NaAsO_2$ and dexamethasone both induced C1 and C2-cells, whereas MMS did not produce C2 cells. The lack of induction of apoptosis by MMS is supported by the results shown in the inserts. The percent annexin V-labelled cells, an indicator of apoptotic cells, showed background levels with MMS but was increased by $NaAsO_2$ and dexamethasone.

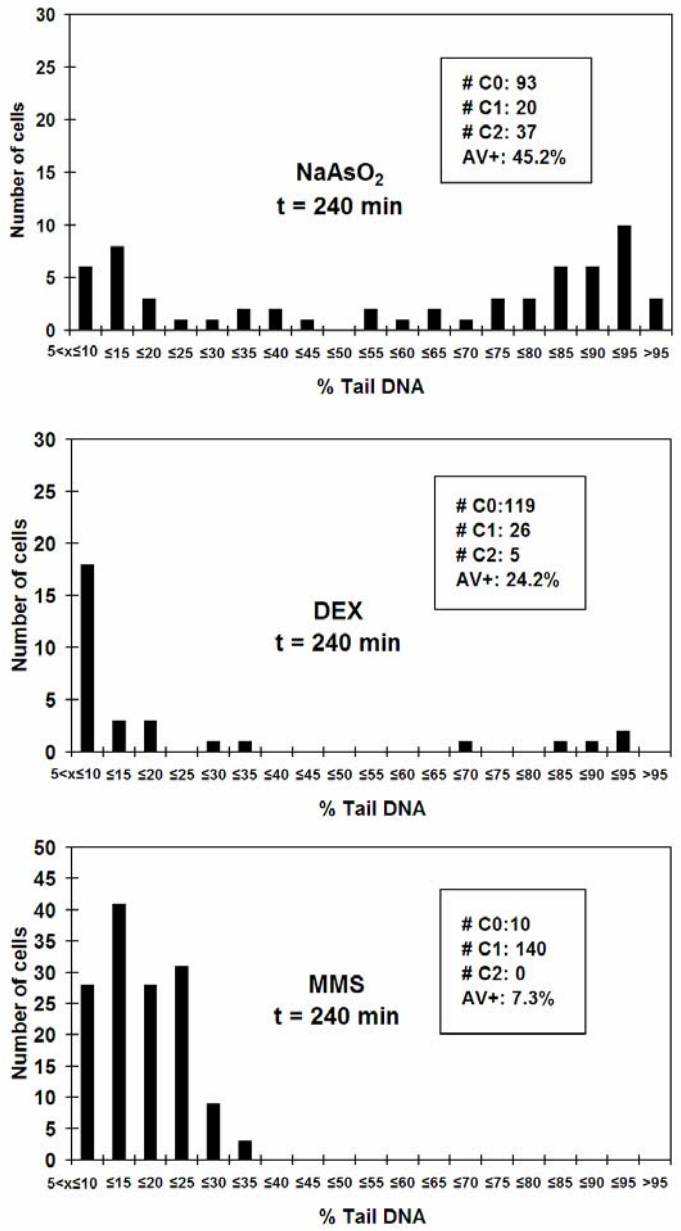


Fig. 19. Distribution of the percentages of migrated DNA (% tail DNA) in the comet assay of 150 L5178Y mouse lymphoma cells pooled from three independent experiments, 4 hours after incubation in medium containing 100 µM sodium arsenite (top panel), 100 µM dexamethasone (center panel), and 200 µM methyl methanesulfonate (bottom panel). The inserts display the number of C0, C1 and C2 cells and the mean percent apoptotic cells (annexin V positive cells; n = 3).

4.4.2 Apoptosis and cell viability analysis

In parallel to the comet assay, apoptosis and cell viability were evaluated by flow cytometry using annexin V / propidium iodide double staining. Positive annexin V staining requires the translocation of the membrane phospholipids phosphatidylserine from the inner to the outer leaflet of the plasma membrane [91].

Thereby phosphatidylserine becomes available for annexin V-binding. This translocation precedes other apoptotic features such as DNA fragmentation, chromatin condensation and loss of plasma membrane integrity. This means that apoptotic cells identified by characteristic morphological features, specifically the condensation of the nucleus, and the formation of apoptotic bodies indicate later stages of the apoptotic process [91]. However, phosphatidylserine detection is also possible during later stages of necrosis. We used annexin V in conjunction with the vital dye propidium iodide, which intercalates with nucleic acids, but only enters into the cell when the membrane integrity is disturbed, observable in late stages of apoptosis and in necrosis but not in early stages of apoptosis.

A typical time series of the flow cytometry analysis (0 to 150 minutes) after $NaAsO_2$ treatment is depicted in Fig. 4. A clear increase of annexin V positive cells was observable 30 minutes after treatment, indicating a rapid onset of apoptosis at the concentration of $NaAsO_2$ used. The number of annexin V positive cells increased further with time. In addition, starting at t = 90, the annexin V positive cells shifted to higher propidium iodide fluorescence, indicating apoptotic cells that increasingly lost their plasma membrane integrity in late stages of the apoptotic process. No increase in necrotic cells was observed.

The development of apoptosis was also monitored by fluorescence microscopy using Hoechst 33258. This DNA stain allows identification of apoptotic cells by characteristic morphological changes, namely chromatin condensation and formation of apoptotic bodies (Fig. 21). In contrast to the annexin V data, apoptotic cells appeared only at 120 minutes after treatment with $NaAsO_2$ (Fig. 22).

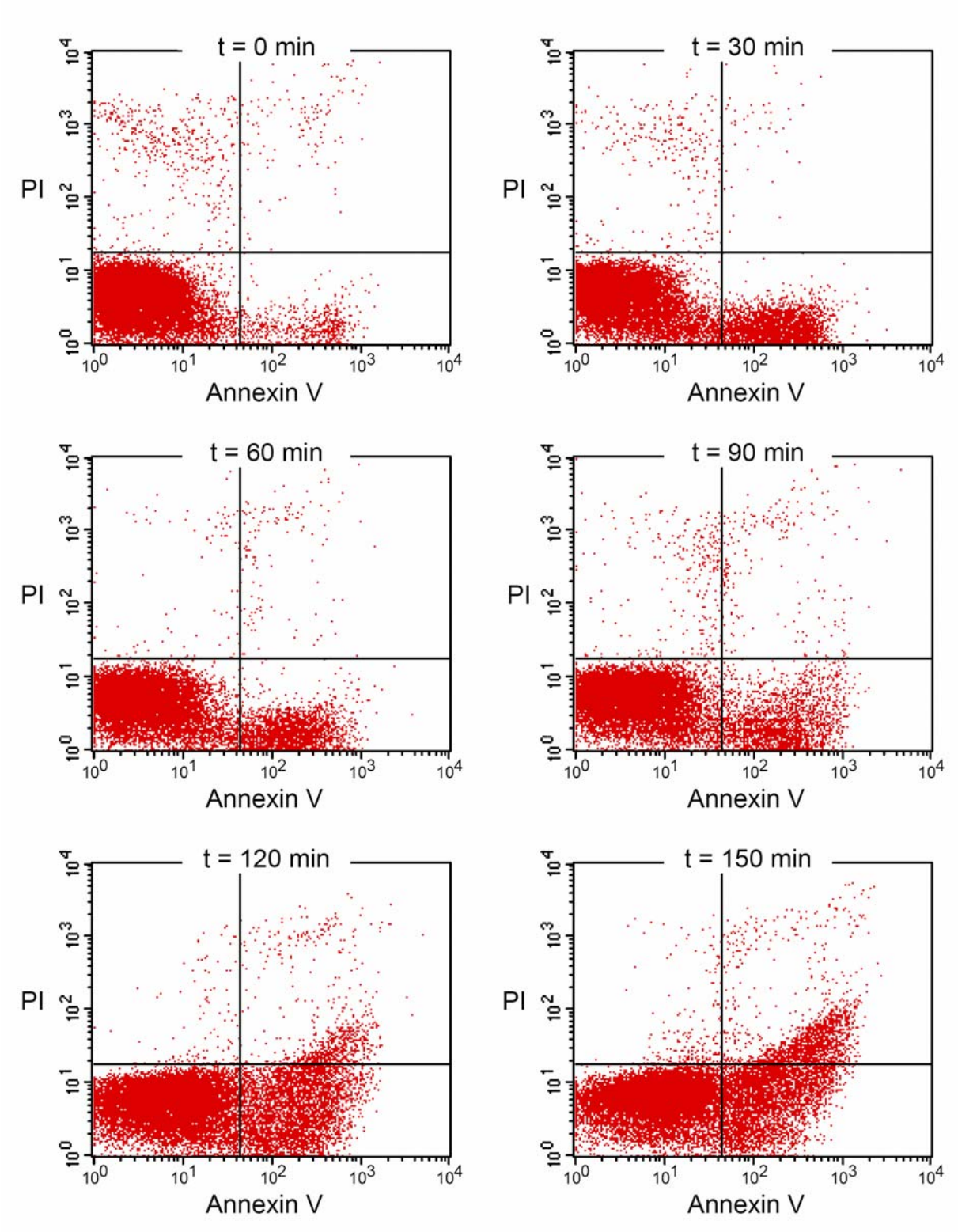


Fig. 20. Time dependent shifts in fluorescence intensity from annexin V (x-axis) and propidium iodide (PI; y-axis) in flow cytometry analyses of L5178Y cells after treatment with 100 µM $NaAsO_2$. The first sample t = 0 was taken immediately after adding $NaAsO_2$. Analyses were conducted in 30-minute intervals. 20,000 events per sample were gated and divided into double negative (lower left; viable cells), annexin-V positive cells (lower right; apoptotic cells), propidium iodide positive cells (upper left; necrotic cells) and double positive cells (upper right; necrotic/apoptotic cells).

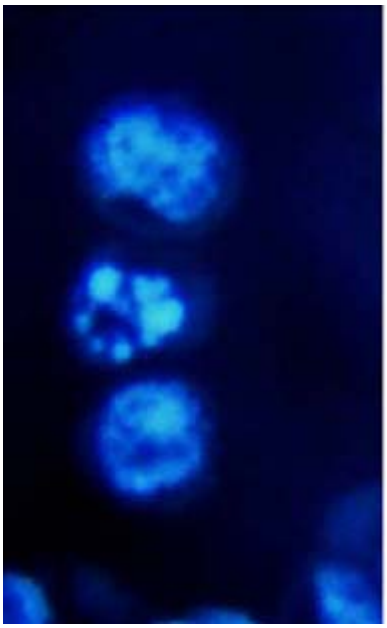


Fig. 21. Microscopic appearance of one apoptotic cell (middle) and two nonapoptotic cells (upper and lower cells) after staining with the fluorescent DNA stain Hoechst bisbenzimide 33258.

4.4.3 Comparative analysis of comet assay and cytotoxicity tests

Fig. 22 compares the time course of all endpoints after addition of $NaAsO_2$. The first observable effect was the increase in annexin-V positive cells. At 90 minutes, the number of ghost cells (C2-cells in the comet assay) increased. Apoptotic cells identified with Hoechst staining appeared at 120 minutes. At later times the number of apoptotic cells stained with Hoechst showed a marked increase while the number of annexin V positive cells and the number of ghost cells increased slowly. The difference is probably due to loss of nuclear fragments during flow cytometry and loss of highly fragmented DNA in the comet analysis, respectively. On the other hand, staining with propidium iodide, often considered to indicate cell viability, did not indicate any effect for up to 180 minutes.

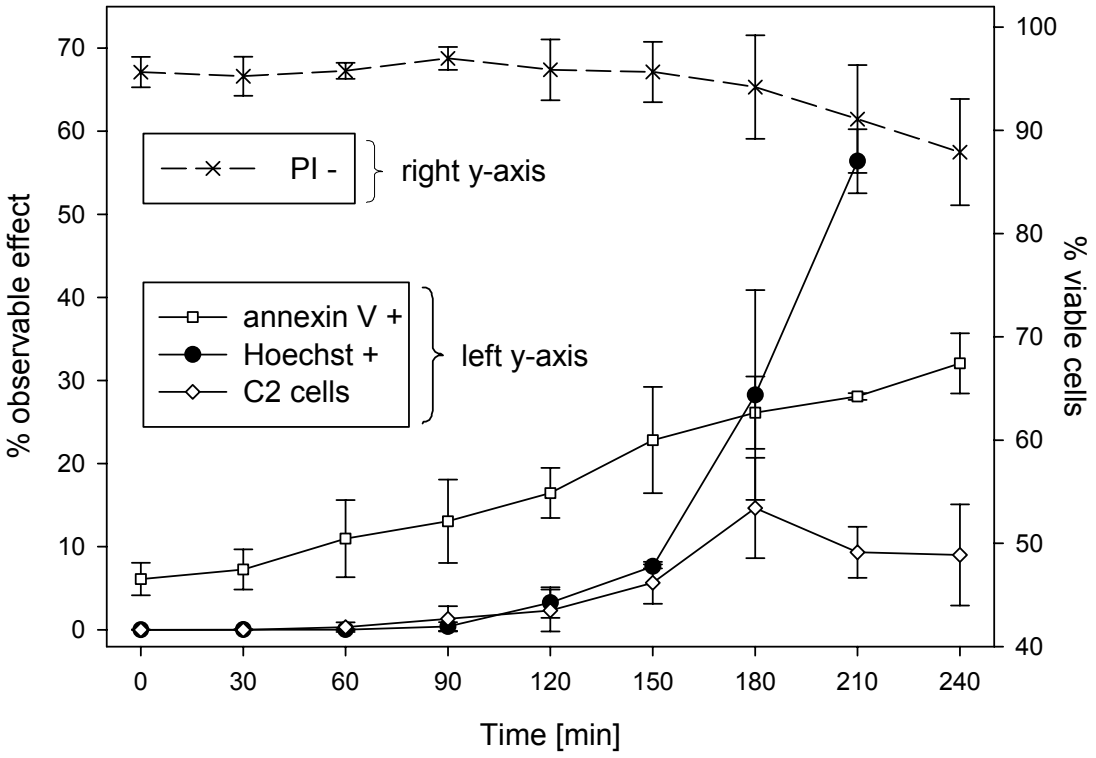


Fig. 22. Four endpoints followed over time to asses the proportion of cells undergoing apoptosis or necrosis after incubation with $NaAsO_2$. Annexin V-positive cells (annexin V +) represent cells undergoing apoptosis. Propidium iodide-negative cells (PI-) are usually considered to represent viable cells. Hoechst-positive cells stand for late apoptotic cells. C2-cells: %Tail DNA > 60 % cells with small or non-existent heads and large diffuse tails. Mean and standard deviation from three independent experiments are displayed.

4.5 Discussion

In comet assay analyses cells showing small heads and high levels of DNA migration are often called "ghost cells" and are thought to represent apoptotic or necrotic cells [79]. An expert panel therefore recommended to exclude these cells from genotoxicity analyses [77]. However, no recommendation was given for a quantitative criterion at what level of DNA migration a cell represents a "ghost cells". For this investigation, >60% Tail DNA was chosen to denote a cell as a "ghost cells" in consideration of values <40%, typically assumed to represent genotoxicity (personal observation). In the here used terminology, these cells with >60% Tail DNA were named C2 cells.

$NaAsO_2$ showed clear time-dependent shift from C0 to C1 to C2 cells when evaluated for the %Tail DNA. A time-dependent effect of $NaAsO_2$ on human leukemia cells had also been observed by Wang *et al.* [92]. Their results show that the comet assay signal increased to a maximum value at about 4 hours and decreased thereafter. The authors concluded that the 4-hour treatment was the optimum time to assess $NaAsO_2$ induced genotoxicity. However, apoptosis was not evaluated so they could not detect a possible influence of apoptosis on the comet assay result.

$NaAsO_2$ effectively triggers apoptosis in numerous cell types and lines. Therefore, the question was addressed whether the C1 cells in our experiments represented cells with genotoxic damage or were on the way to undergo apoptosis. The C1 cells appeared prior to the C2 cells and their number decreased in parallel with an increase in the number of C2 cells. Annexin V staining, a marker of early apoptosis showed an increase in apoptotic cells in parallel with an increase in C1 cells (see square symbol in Fig. 22 and C1 cells in Fig. 18). Although we cannot entirely exclude genotoxicity in all C1 cells, we postulate that a portion of the C1 cells were precursors of C2 cells and did not indicate genotoxicity of $NaAsO_2$. Hence, C1 cells may be formed by two different mechanisms, genotoxicity and apoptosis. This also means that a comet assay result could falsely be interpreted as indicative of genotoxicity even if ghost cells are excluded from the analysis.

To test whether genotoxicity can be discriminated from apoptosis the effects of positive control chemicals was investigated, MMS as a genotoxic agent (methylating agent) and dexamethasone as a non-genotoxic apoptosis inducer. Dexamethasone triggers apoptosis in L5178Y cells by binding to the glucocorticoid receptor and

transcription of specific death genes and is devoid of genotoxic properties [89] [90]. MMS is a methylating agent frequently used as positive control in genotoxicity analysis. Treatment of cells with MMS produced no C2 cells within 240 minutes and no increase in annexin V positive cells. In contrast, dexamethasone induced C1 within 120 minutes (data not shown) and C2 cells within 240 minutes. Additionally, the number of annexin V positive cells was increased. This supports our hypothesis that a subpopulation of C1 cells represented precursors of C2 cells and were early apoptotic cells. A bimodal distribution of DNA migration had also been observed by [90] after 3 hours treatment of L5178Y mouse lymphoma cells with dexamethasone, but no longer after 24 hours.

To safeguard against misinterpretation of comet assay results due to cell death, measurement of cell viability has been recommended [77]. Henderson et al. (1998) [93] reported that a cell viability greater than 75% (based on exclusion of vital dyes e.g. propidium iodide or trypan blue) assessed at the time of comet analysis should be obtained. However, it was recently shown that the quantity of cells undergoing apoptosis or necrosis after treatment with $NaAsO_2$ was underestimated when tested with vital dyes too soon after the addition of the chemical [88]. This is due to the fact that early apoptotic cells still maintain their membrane integrity and do not stain with e.g. trypan blue or propidium iodide. Indeed, we found a proportion of propidium iodide-positive cells <10% at times where the early apoptotic marker annexin V indicated appreciably greater proportions of dying cells.

The comet assay studies on $NaAsO_2$ published so far found positive responses indicating genotoxicity using various concentrations (2.5 µM – 10 mM) but only one fixed period of incubation (30 minutes to 8 hours) [82-85,94]. It was demonstrated by evaluating frequency histograms of %Tail DNA that the effects of $NaAsO_2$ in the comet assay were strongly time-dependent and influenced by the onset of apoptosis.

To evaluate DNA damaging effects of $NaAsO_2$ using the comet assay a single exposure time runs the risk of misinterpretation, even if the accompanying vital dye test indicates sufficient viability. The comet assay signal, however, can be interpreted as a measure for DNA strand breaks (in the alkaline version also for abasic sites), if a simultaneous early endpoint for apoptosis like annexin V staining indicated no increase in apoptosis or necrosis at the time of comet analysis.

5 Biological significance of DNA alkylation

5.1 Background

5.1.1 DNA adducts: background vs. induced

DNA in living cells is constantly damaged even in the absence of any exposure to genotoxic carcinogens by a specific treatment [17]. At all time there is a (steady-state) level of DNA damage present, referred to as background DNA damage, even though cellular processes like DNA-repair constantly reduce the amount of damage. Due to analytical developments the detection and quantification of DNA adducts is achievable at continuously decreasing levels with increasing specificity and numerous DNA adducts with background occurrence were identified [65]. Highly specific LC-MS/MS methods allow quantification of DNA adducts at background levels under best possible conditions even down to 1 modified nucleoside in 10^9 normal nucleosides [30,53]. The total background level for DNA damage can be as high as 1 modification per 10^5 normal nucleotides [18]. This level was estimated based on a large list of DNA damaging sources of endogenous and unavoidable exogenous origin. In view of this remarkable level (note: 1 per 10^5 unmodified nucleotides is equivalent to 10.000 DNA lesions per one diploid mammalian cell) a minute increase of 1 adduct per 10^9 from an exposure might be measurable but the biological consequences may vanish in the background and its normal fluctuation.

On this basis a discussion was stimulated on the biological significance of small exposure-related increments of DNA adducts and their relevance to human risk assessment of chemicals [95-101]. Key questions are on the biochemical and biological consequences of different adducts, the requirements for the primary DNA damage to be expressed as heritable genetic changes and the quantitative relationship between background adduct levels and exposure-related increments i.e. to what extent do increments in adducts result in increments in subsequent endpoints of genotoxicity and mutagenicity. Here, this question was addressed *in vitro* by measuring adducts induced by alkylating agents and simultaneous investigation of endpoints of genotoxicity and mutagenicity.

5.1.2 DNA adducts induced by methylating agents

In general DNA-reactive carcinogens not only generate one single DNA adduct but show different affinity to react with different nucleophilic sites in DNA. For alkylating agents there are about one dozen of cites in double stranded DNA which are targeted. The different proportions of adducts at oxygen versus nitrogen in DNA depend upon the nature of the reactive moiety [11]. Alkylation of double-stranded DNA by most alkylating agent occurs predominantly at the 7(N)-position of guanine. This results in a formal positive charge, which destabilizes the N-glycosidic bond between guanine and the 2′-deoxyribose and facilitates depurination to yield 7-methylguanine (7-mG) and an apurinic site [63]. Apurinic sites may be hydrolyzed under cellular pH conditions to DNA strand breaks. Moreover, apurinic sites may give rise to mutations, if they remain unrepaired and a DNA polymerase passes by this lesion (A-rule) inserting preferably an adenosine opposite to a non instructive abasic site [13,63]. Alkylations at O^6 of guanine and O^4 of thymine, are considered in particular to be responsible for the induction of base pair substitution mutations by mispairing during DNA replication. A mismatch of O^6-mdGuo with thymine may be repaired by mismatch repair (MMR), but intermediate stages of MMR like DNA strand gaps may lead to malfunctioning DNA replication forks that possibly give rise to double strand breaks [102,103]. The distribution of DNA-methylation products appears to be an important factor for the biological effects generated by methylating agents, since the relative carcinogenic potency of these agents seems to correlate with the reagent′s ability of bind to oxygen sites [104].

Two methylating genotoxins, methyl methanesulfonate (MMS) and N-methyl-N-nitrosourea (MNU), with different modes of action were used to investigate background DNA damage and dose-related increments in L5178Y mouse lymphoma cells (Fig. 23). Background methylations could result from aberrant methylation of DNA by S-adenosyl-L-methionine (SAM), the physiological methyl group donor for the enzymatic methylation of cytosine to 5-methylcytosine [19]. MMS reacts preferably through a S_N2-type mechanism whereas MNU predominately reacts via an mechanism of S_N1-type via a highly reactive methyl diazonium ion (Fig. 23).

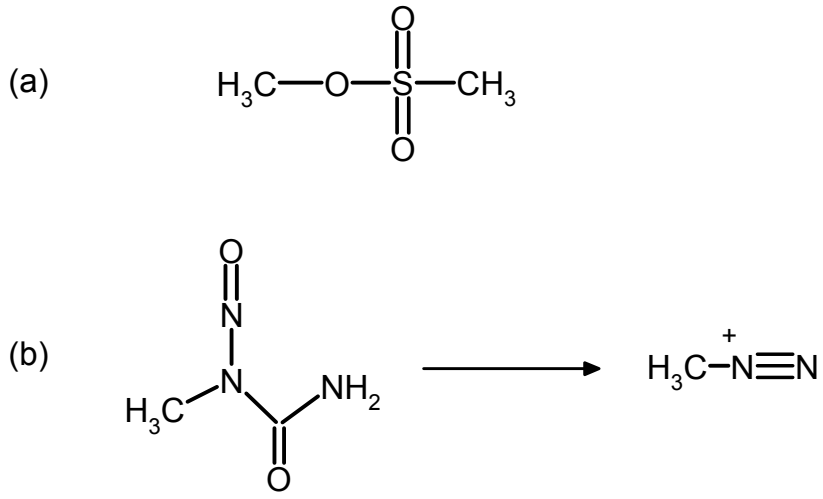


Fig. 23. Chemical structures of the methylating agents (a) methyl methanesulfonate (b) N-methyl-N-nitrosourea and the intermediate methyl diazonium ion, involved in the reaction mechanism of S_N1-type.

Large batches of L5178Y mouse lymphoma cells were treated with MMS or MNU at different concentrations. The formation of guanine methylation was determined by LC-MS/MS (7-mG and O^6-mdGuo). In parallel, using the same population of treated cells three different genotoxicity assays were performed. (i) The comet assay, (ii) the micronucleus test and (iii) the mouse lymphoma assay to determine DNA breakage, chromosomal damage and gene mutations, respectively. The comet assay in its alkaline version detects DNA single- and double-strand breaks, as well as alkali-labile sites (e.g. apurinic sites) by converting these lesions into DNA strand breaks. The micronucleus test detects chromosomal damage, the mouse lymphoma assay determines forward mutations at the thymidine kinase locus.

The various endpoints were correlated with each other, including comparison of slopes at low dose. At the cellular level chromosomal aberrations or mutations are

assumed to be early alterations on the pathway from a normal cell to a tumor cell, thus representing biochemical or biological endpoints of genotoxicity that can relate induced DNA-adduct levels to the process of tumorigenesis.

5.2 Materials and Methods

5.2.1 Cell culture and treatment

The L5178Y mouse lymphoma cell line, clone 3.7.2c (obtained from W. J. Caspary, NIEHS, USA) was used throughout all experiments. Ongoing cultures were maintained at 37°C in a humidified atmosphere with 5% (v/v) CO_2 in normal growth medium (RMPI 1640 containing antibiotics (penicillin, streptomycin), 2 mM L-glutamine, 20 mg/ml sodium pyruvate and 10% (v/v) horse serum). Prior to each experiment an exponentially growing culture was incubated for 24 h in THMG medium (culture medium containing 3 µg/ml thymidine, 5 µg/mL hypoxanthine, 0.1 µg/mL methotrexate and 7.5 glycine). This selective medium permits the normal growth of $tk^{+/-}$ cells while killing $tk^{-/-}$ cells [105], thereby reducing the background frequency of spontaneous $tk^{-/-}$ mutants. Then the cells were transferred to THG medium (THMG medium without methotrexate) for 48 h. After another 7 days of growing in normal growth medium, 2.8 x 10^7 cells per dose were seeded (3 x 10^5 cells/ml) for treatment with MMS or MNU. Stock solutions of MMS or MNU were prepared in dimethylsulfoxide (DMSO) immediately before addition to the cells (final concentration of DMSO below 0.1%). After 4 h of incubation, the cells were washed with growth medium and split up for concurrent performance of DNA adduct quantification, comet assay analysis, the micronucleus test, a cell viability assay and the thymidine kinase mutation assay.

5.2.2 DNA isolation and quantification of DNA adducts

Genomic DNA was extracted from approximately 2.4 x 10^7 cells using a DNA isolation kit (AXG 100, Nucleobond®, Macherey-Nagel, Düren, Germany) following the users manual. The columns were eluted twice resulting in two DNA fractions. The DNA from the first elution and precipitation step (yield ~ 100 µg of DNA) was used for the analysis of the 2′-deoxyribonucleosides O^6-mdGuo, 8-oxodGuo and εdAdo using liquid chromatography tandem mass spectrometry with column switching as

described in detail before in [106] and in chapter 3. The DNA from the second elution step (same volume of elution buffer as in step one, yield: ~ 20 to 40 µg of DNA) was used for the analysis of 7-mG as described in chapter 3.

5.2.3 Comet assay analysis

The alkaline version of the comet assay was performed according to recommendations published elsewhere [77] using approximately 1 x 10^6 L5178Y mouse lymphoma cells (protocol details are provided in chapter 4).

5.2.4 Micronucleus test

The micronucleus test was conducted based on published recommendations [37]. After one additional washing step with growth medium a sample of 1 x 10^6 cells was suspended in growth medium containing cytochalasin B (5 µg/ml) and incubated for another 18 h. Then the cells were washed with PBS buffer, collected by centrifugation (200 x g, 5 min) and placed on glass slides by cytospin centrifugation (200 x g, 5 min). For fixation the slides were immersed in methanol at -20°C. Immediately before analysis by fluorescence microscopy the slides were immersed for 5 min in a solution of acridine orange (62.5 µg/ml) in Sørensen buffer (15 mM Na_2HPO_4 and 15 mM KH_2PO_4, pH 6.8) and washed twice with Sørensen buffer. For each concentration two replicate slides were prepared and 1000 binucleated cells (BN) per slide were scored for micronuclei-containing binucleated cells.

5.2.5 Mouse lymphoma thymidine kinase assay: 96-well version

The 96-well version of the mouse lymphoma thymidine kinase mutation assay was employed according to published recommendations [43,107]. After harvesting and washing, the treated cells were suspended at 2 x 10^5 cells /ml in normal culture medium for a total expression period of 48 hours, during which the TK-deficient phenotype can be expressed. After 24 h cell density was controlled (using a Coulter counter) and diluted again to 2 x 10^5 cells /ml. At the end of the expression period cell density was counted and adjusted to 1 x 10^4 cells/ml suspended in 50 ml of normal culture medium containing 3 µg/ml of the toxic thymidine analogue 2′-deoxy-5-trifluoromethyl-uridine (trifluorothymidine; TFT) for mutant selection plating (density:

10,000 cells/ml). Before TFT was added 32 µl of the cell suspension was diluted to 40 ml of normal growth medium (density: 8 cells/ml) for viability plating. The cell suspensions were dispensed at 0.2 ml/well on 96-well plates (duplicates) using a multichannel pipette resulting in an average of 2000 and 1.6 cells/well for mutant selection and viability plates, respectively. The plates were incubated for 12 days at 37°C in a humidified atmosphere with 5% (v/v) CO_2. Wells containing no viable clone were identified by eye using background illumination and counted.

Calculations: The key parameter of the 96-well procedure is the number of negative wells on each plate [43,108]. From the zero term of the "Poisson distribution" the probable number of clones/well on a plate with a number of empty wells (EW) out of a total of 192 (2 x 96) wells, is given by:

$$P = -\ln\left(\frac{\mathrm{EW}}{192}\right)$$

If an average of 1.6 cells (2000 on mutant selection plates) were plated the plating efficiency (PE) is given by:

for viability plates: $$\mathrm{PE}_{(\mathrm{viable})} = \frac{P_{(viable)}}{1.6}$$

and for mutant selection plates: $$\mathrm{PE}_{(\mathrm{mutant})} = \frac{P_{(mutant)}}{2000}$$

The mutant frequency (MF) is given by**:** $$\mathrm{MF} = \frac{\mathrm{PE}_{(\mathrm{mutant})}}{\mathrm{PE}_{(\mathrm{viable})}} \times 10^6$$

The mutant frequency was expressed as "mutants per 10^6 viable cells".

Suspension growth (SG) was calculated as: $$\mathrm{SG} = \frac{\mathrm{D}_1}{\mathrm{D}_0} \times \frac{\mathrm{D}_2}{\mathrm{D}_{1/0}}$$

D_0 : cell density set up on day 0 after treatment
D_1: cell density on day 1
$D_{1/0}$: cell density set-up on day 1
D_2: cell density on day 2

Relative Suspension Growth (RSG): $RSG = SG_{(conc.)}/SG_{(control)}$

5.3 Results

5.3.1 DNA methylation by MMS and MNU

Fig. 24 shows the dose-dependent formation of the DNA adducts 7-mG and O^6-mdGuo determined in L5178Y mouse lymphoma cells. The cells were treated at equimolar concentrations of MMS and MNU. Means and standard deviations from 3 completely independent experiments per agent are shown. Background (solvent control) levels of 7-mG were determined at 1435 ± 77 adducts per 10^9 normal nucleotides (nt) (MMS series) and 1349 ± 479 (MNU series). Dose-dependent treatment resulted in a linear increase up to 100 µM for both agents. Further increase extended the linearity for MNU, while a sigmoid dose response was observed with MMS. At 200 and 300 µM MMS the variation of 7-mG appeared very large (Fig. 24), however this impression is only visual. The coefficients of variation (CVs) for 7-mG were not particularly large. At 0, 50, 100, 200, and 300 µM MMS they were 5, 41, 51, 68, and 61 percent, respectively. For O^6-mdGuo the respective CVs were (exclusive of 0 µM MMS) 60, 76, 53, and 39 percent. Regarding slopes of the dose responses, 7-mG induction was more effective by MMS than by MNU.

The background level of O^6-mdGuo was below the limit of detection in the standard samples of 100 µg of DNA (40 adducts per 10^9 nt, see chapter 2). In separate experiments, DNA was isolated from L5178Y mouse lymphoma cells (DMSO control, grown to a density of 10^6 cells per ml) and using 400 µg of DNA per HPLC run a background for O^6-mdGuo was measured at 32 ± 8 adducts per 10^9 nt. This is indicated by a cross in brackets in Fig. 24b. Treatment with MMS or MNU at 50 µM resulted in a clearly measurable level of O^6-mdGuo with means of 178 ± 106 and 701 ± 191 adducts per 10^9 nt, respectively. Further increase in dose produced clear linearity for O^6-mdGuo for both MMS and MNU. Comparing the two methylating agents, MNU produced in a steeper increase than MMS, which was in obvious contrast to the situation with 7-mG.

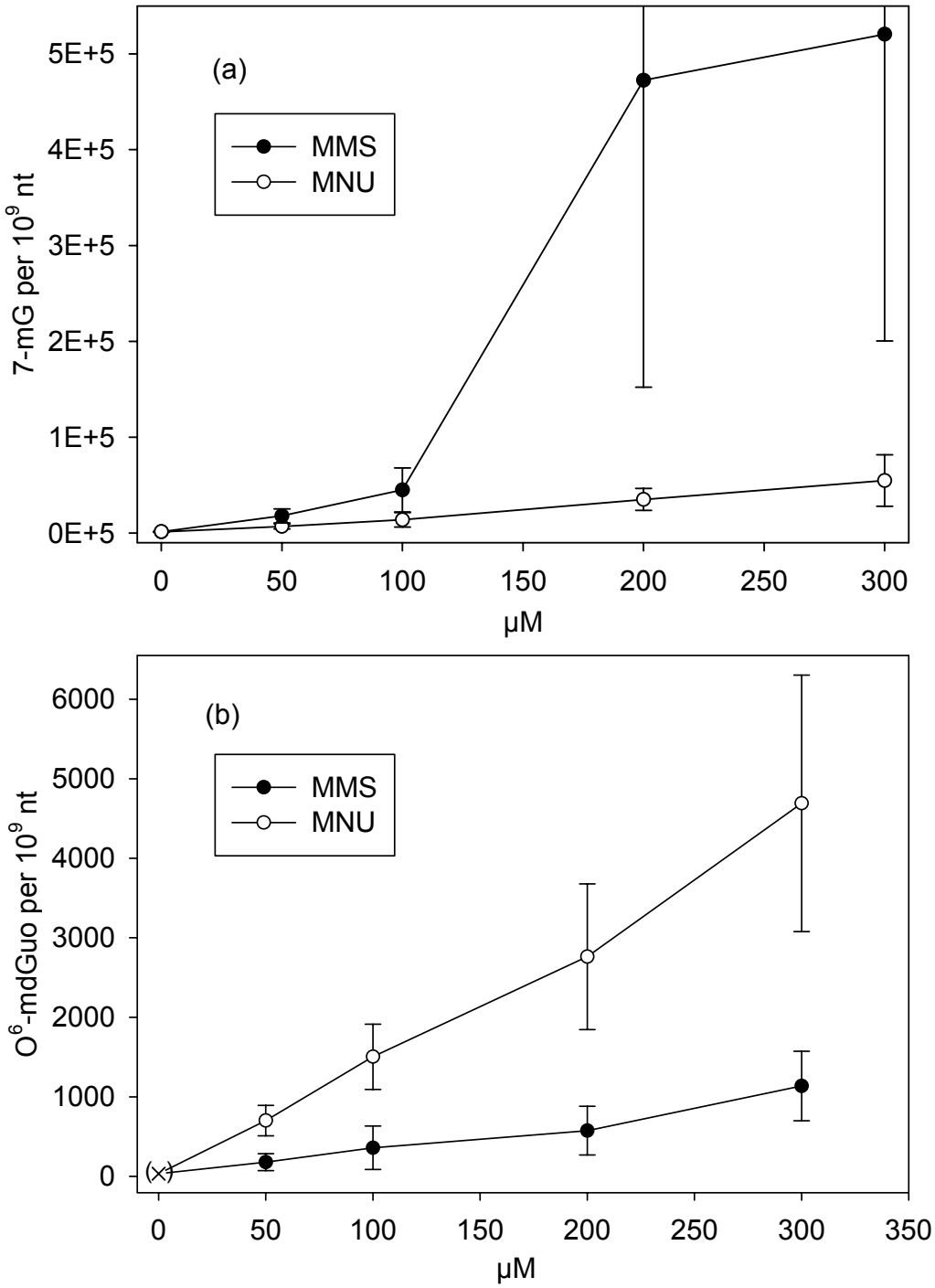


Fig. 24. Dose-response relationships for the formation of (a) 7-methylguanine (7-mG) and (b) O^6-methyl-2′-deoxyguanosine (O^6-mdGuo) expressed per 10^9 nucleotides (nt) measured in L5178Y mouse lymphoma cells treated with methyl methanesulfonate (MMS) or N-methyl-N-nitrosourea (MNU). The cross in brackets indicate that the O^6-mdGuo background was determined in separate experiments. Means and standard deviations of three independent sets of experiments are given.

5.3.2 DNA adduct profile: Relative reactivity of MMS and MNU

The ratio of 7-mG to O^6-mdGuo was calculated for all concentrations and listed in Table 3. For MNU the ratio was about 10-fold smaller at 50 and 100 µM concentration when compared to MMS. At 200 and 300 µM the difference was even 50 and 30-fold. This different distribution of DNA methylation products reflects the different mechanism by which the ultimate methylating moiety of MMS and MNU reacts with DNA. MMS reacts with nucleophilic sites in DNA through a S_N2-type mechanism whereas MNU decomposes to a diazonium ion that methylates DNA by S_N1 reaction. Due to different consequences (depurination versus mispairing) of the different adducts the different distributions of methylation products in DNA are proposed to be differently relevant for biological consequences.

Table 3
Ratios of 7(N) to O^6-methylation of guanine in DNA of L5178Y mouse lymphoma cells treated with different methylating agents

	7-mG/O^6-mdGuo ratio	
[µM]	MMS	MNU
50	113 ± 45	11.1 ± 5.9
100	148 ± 46	10.5 ± 7.4
200	767 ± 307	14.4 ± 7.5
300	463 ± 304	14.1 ± 11.2

MMS and MNU clearly showed different relative reactivity towards the oxygen versus nitrogen of guanine. However, both agents MMS and MNU showed a relative preference to introduce a methyl group at the 7(N) position than for oxygen, but MNU less so than MMS. MNU resulted in relatively greater amounts of O^6 methylation, compared to MMS, treatment. The 7-mG to O^6-mdGuo ratio did not change with dose of MNU, while it was dose dependent for MMS, and showed an increase above 100 µM. This shift is also reflected by the observation of the sigmoid dose response for 7-mG, while O^6-mdGuo increased dose-linear with MMS (Fig. 24).

5.3.3 DNA breakage and chromosomal damage

Fig. 25 shows the results of the comet assay and micronucleus test. Background levels were reproducibly measurable in both assays and were in the range of 1.6 to 6.8 % Tail DNA and 11 to 32 micronuclei per 10^3 binucleated cells (MN/10^3 BN), respectively. The values were in line with background values typically observed in these genotoxicity assays using L5178Y mouse lymphoma cells, although usually smaller batches of cells are used. Treatment-related increases were expressed relative to background. With increasing concentration of both MMS and MNU the comet assay signal increased dose linearly. For MMS the increase above control was steeper as compared to MNU indicating that MMS induced DNA single-strand breaks and/or apurinic sites with higher potency than MNU. In the MN test, MNU treatment did not show an obvious dose-related effect, but linear regression analysis with all data points produced a significant slope (Table 5). MMS treatment resulted in a clear increase of MN frequency, indicating a higher clastogenic potential. The comet assay was conducted immediately at the end of the incubation period, representing the situation at the time of adduct analyses. The micronucleus test adds a period of 18 hours for nuclear division at the end of the treatment period. This period provides time for recovery, e.g. by DNA repair processes. Saturation of these processes at higher doses can then introduce sublinearity. Indeed the dose response for MMS indicated some upward convex trend for the induction of micronuclei (Fig. 25).

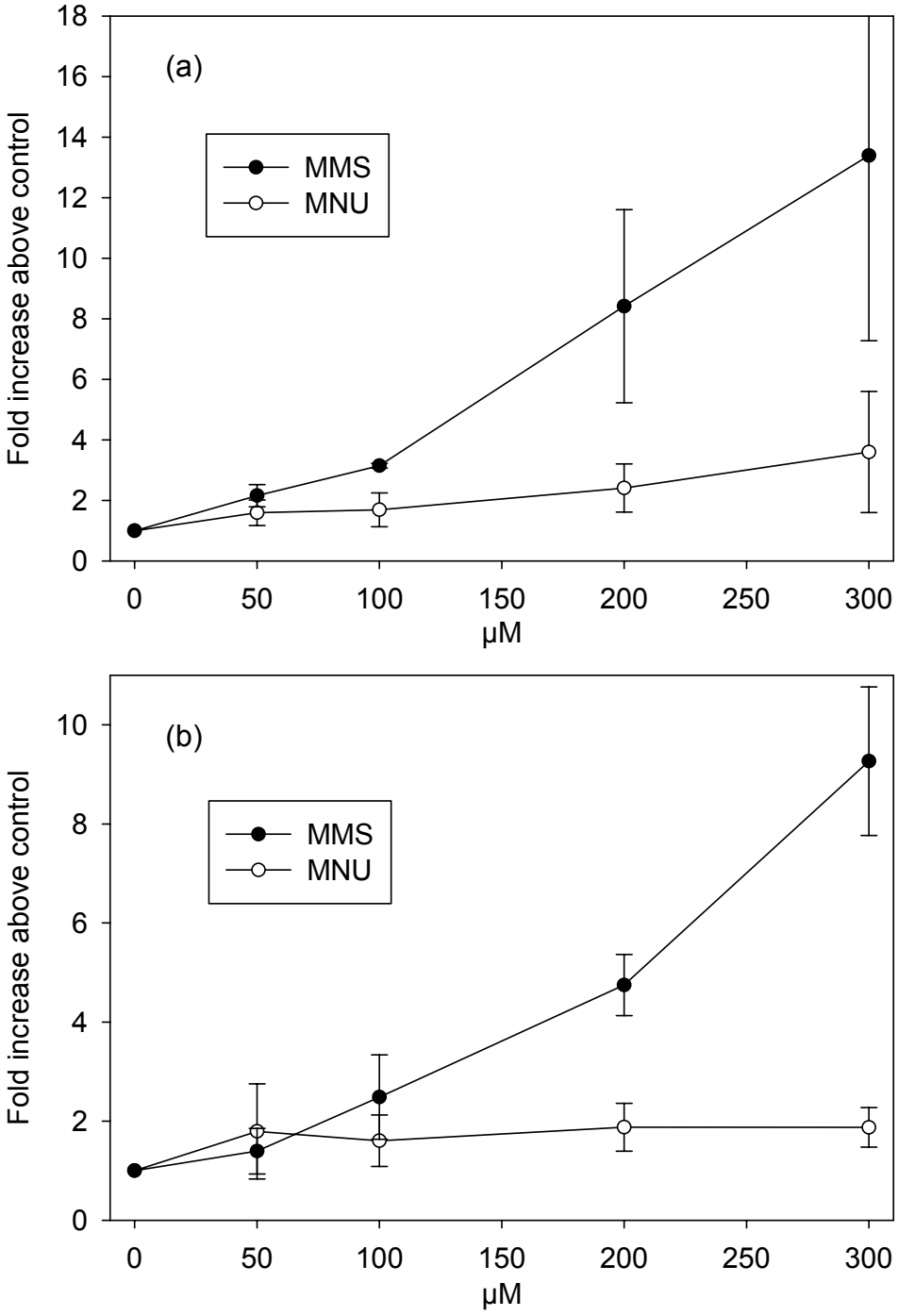


Fig. 25. Dose response for DNA breakage after incubation of L5178Y mouse lymphoma cells expressed in relative terms above background measured in L5178Y mouse lymphoma cells treated with methyl methanesulfonate (MMS) or N-methyl-N-nitrosourea (MNU). (a) comet assay analysis, (b) micronucleus test. Means and standard deviations of three independent sets of experiments are given.

5.3.4 Mouse lymphoma thymidine kinase assay

Fig. 26 shows the mutant frequency assessed with the mouse lymphoma assay in parallel to the DNA methylations (Fig. 24), DNA breakage and chromosomal damage (Fig. 25). The cell culture had been cleansed by pretreatment with methotrexate in order to reduce the number of spontaneous mutants $tk^{-/-}$ in the culture. The sensitivity of the assay to detect low-dose effects of MMS was significantly improved by this procedure (data of preliminary experiments; not shown). The cleansing procedure was found to be cytotoxic per se (increased necrosis and apoptosis; data not shown), hence it was not recommended to use the cleansed cultures directly after the procedure. On the other hand, background $tk^{-/-}$ mutants accumulated again after the cleansing, which reduced the sensitivity continuously. Therefore, a time window for experiments after the cleansing procedure was determined for the investigation of small mutagenic effects with high sensitivity. Under our experimental conditions this time window was determined to be one to two weeks after cleansing [109]. Therefore, the large series experiments with MMS and MNU were started always at day 7 after the cleansing procedure.

A background mutant frequency was reproducibly measurable in all solvent control samples at 78 ± 33 (MNU series) and 64 ± 9 (MMS series). Both MMS and MNU resulted in a dose-dependent, apparently linear increase in mutant frequency. As opposed to the comet assay and micronucleus tests, MNU was clearly more potent than MMS. Suspension growth was used as parameter to demonstrate sufficient cell proliferation during the mutant expression period. For MMS and MNU the relative suspension growth values of 98 ± 11% and 85 ± 8% were determined at concentrations used for linear regression analysis (50 and 100 µM). These values were acceptable according to the criteria specified for the mouse lymphoma assay [43,110,111].

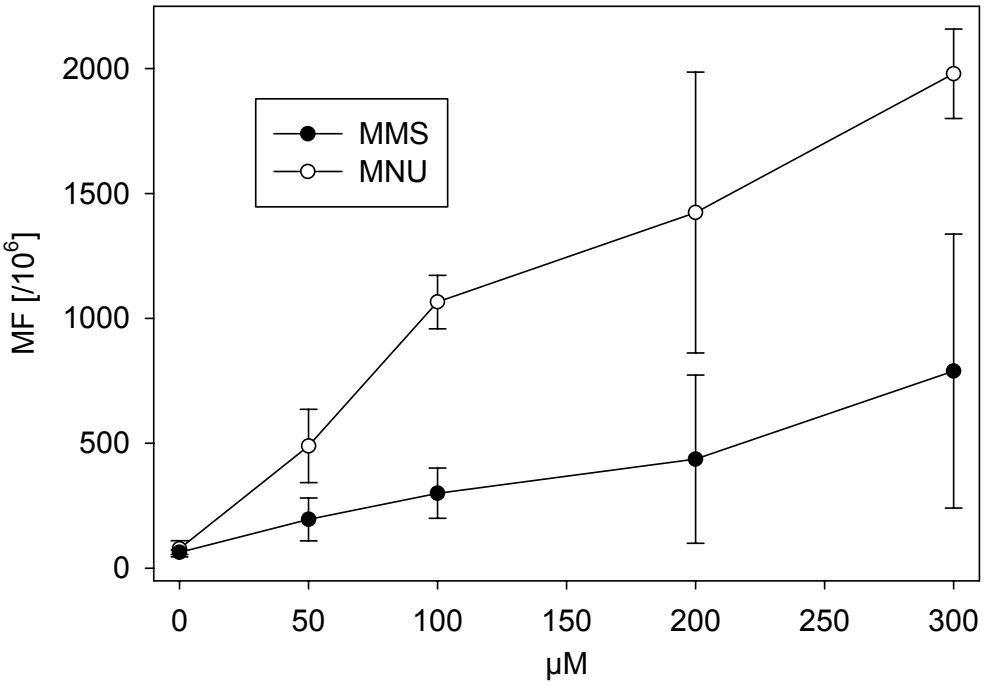


Fig. 26. Dose response for $tk^{-/-}$ mutant frequency (MF [/10^6]), measured in L5178Y mouse lymphoma cells treated with methyl methanesulfonate (MMS) or N-methyl-N-nitrosourea (MNU). Means and standard deviations of three independent sets of experiments are given.

5.3.5 Cell viability

Apoptotic and necrotic cells were determined by flow cytometry. Cells were stained with propidium iodide in conjunction with annexin V-Fluos. Cells negative for both stains were considered as viable cells (compare subsection 4.3.5). In Fig. 27 the decrease in viable cells due to MMS (left chart) and MNU (right chart) is shown. To exclude cytotoxicity as a confounding factor in comet assay analyses cell viability should be greater than 75%, assessed at the time of analysis [77,93]. Further it is recommended to exclude the induction of early apoptosis as discussed in chapter 4. Here, cell viability determined with propidium iodide/annexin V double staining was always greater than 80% at the end of the 4 h incubation period over the entire range of MMS and MNU concentrations applied.

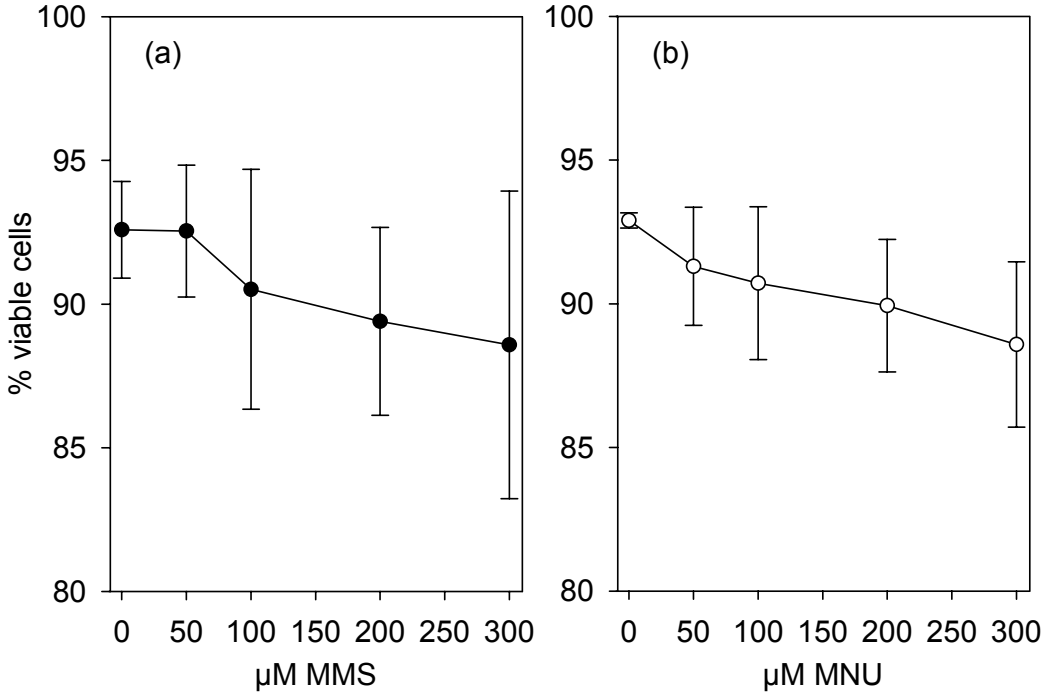


Fig. 27. Dose dependent decrease of viability in L5178Y mouse lymphoma cells treated with (a) methyl methanesulfonate (MMS) or (b) N-methyl-N-nitrosourea (MNU). After staining cells with propidium idodide in conjunction with annexin V the percentage of viable cells (cells negative for both stains) was determined by flow cytometry.

5.3.6 Oxidative DNA modifications

Exposure to methylating agents not only results in methylation specific adducts but may also influence the levels of other endogenous DNA lesions. Two working hypotheses can be named. First, methylation of glutathione could result in an increase in oxidative stress which could result in direct and indirect DNA alterations such as 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxo-dGuo) and 1,N^6-etheno-2′-deoxyadenosine (εdAdo), respectively [112]. Second, base excision repair (BER) that mainly acts on 7-mG adducts is a highly efficient DNA repair pathway processing various lesions by the action of substrate specific glycosylases. Since the BER pathways acting on different lesions also share different enzymes (e.g. DNA ligases), high levels of 7-mG adducts could affect the repair of other endogenously formed lesions. Based on these hypotheses, MMS would be expected to be more effective than MNU because of the higher proportion of glutathione methylation and 7-mG formation. The oxidative DNA lesions 8-oxodGuo and εdAdo were measured by LC-MS/MS simultaneously [106]. Fig. 28 shows the results. Background levels of both 8-

oxodGuo and εdAdo were clearly measurable but no significant increase was seen after any treatment, indicating that neither treatment with MMS nor with MNU influenced processes of direct oxidation of DNA or lipid peroxidation within the range of concentrations used.

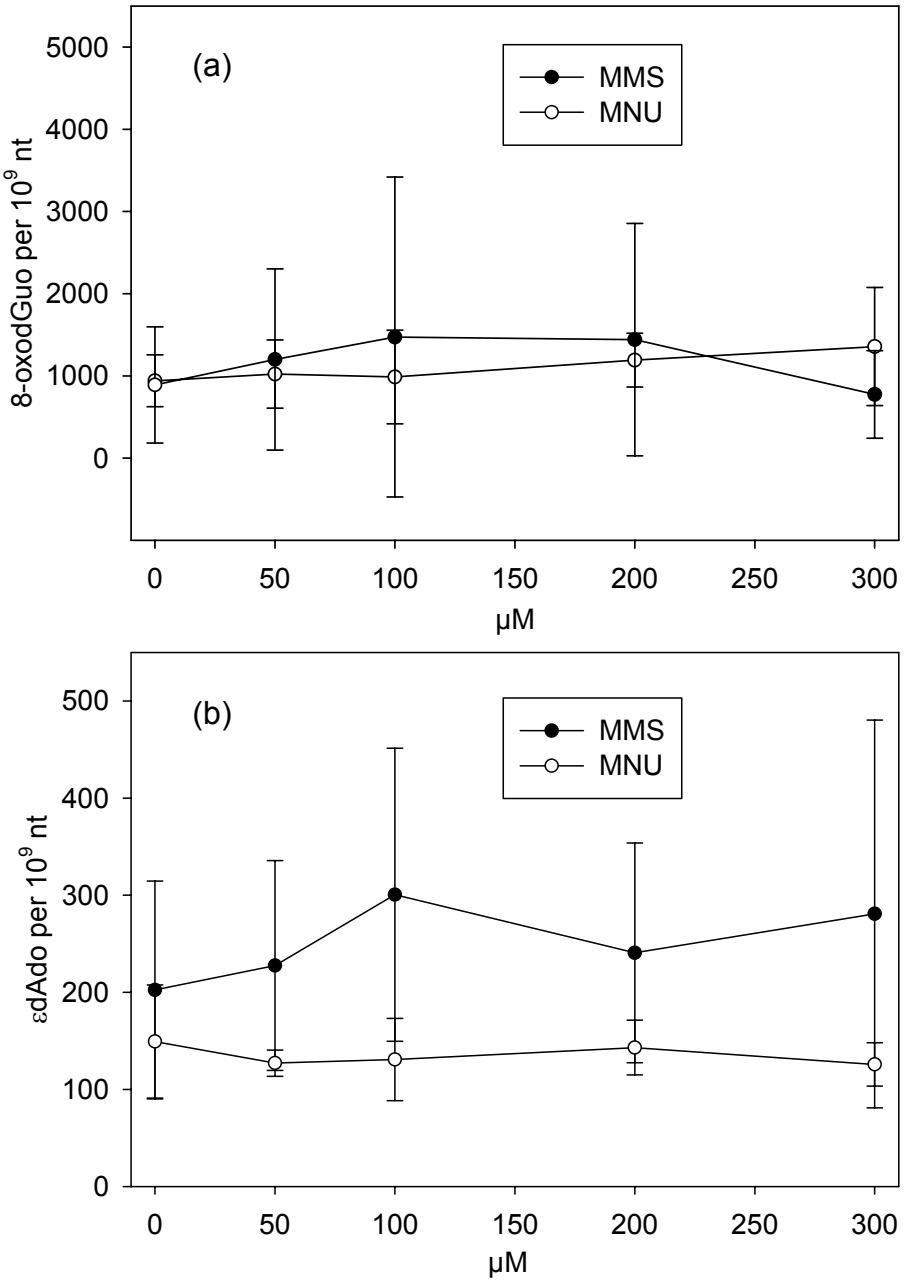


Fig. 28. Levels of (a) 8-oxodGuo and (b) εdAdo in L5178Y mouse lymphoma cells after treatment with different doses of methyl methanesulfonate (MMS) or N-methyl-N-nitrosourea (MNU). Levels are given as adducts per 10^9 normal nucleotides (nt).

5.3.7 Comparison of DNA adducts with other endpoints of genotoxicity

The increases observed in the different endpoints were expressed as relative increments in multiplicative terms to background and are shown in Table 4. Two methylation specific DNA adducts (7-mG and O^6-mdGuo) and 3 biological endpoints of genotoxicity were assessed in parallel. The relative increments were calculated for each experiment and mean values of three completely independent series are given. Standard deviations were not included for sake of clarity. For variability of the different endpoints see the respective graphs (Figs. 2 – 4).

Table 4
Dose-related increments expressed in multiplicative terms (fold induction) to background for 7-methylguanine (7-mG), O^6-methyl-2′-deoxyguanosine, (O^6-mdGuo), and for the biological endpoints comet assay, micronucleus (MN) test and mutant frequency (MF). All endpoints were measured in L5178Y mouse lymphoma cells treated with methyl methanesulfonate (MMS) or N-methyl-N-nitrosourea (MNU).

	DNA adducts				Biological endpoints					
	7-mG		O^6-mdGuo		comet assay		MN		MF	
[μM]	MMS	MNU	MMS	MNU	MMS	MNU	MMS	MNU	MMS	MNU
50	12	5.2	5.6	35	2.2	1.6	1.4	1.8	3.0	6.5
100	31	10	11	75	3.1	1.7	2.5	1.6	4.7	15
200	331	15	18	104	8.4	2.3	4.8	1.9	6.4	18
300	362	27	36	138	13	2.4	9.2	1.9	11.7	29

5.3.7.1 Induction of 7-mG versus comet assay and micronucleus test

For both agents MMS and MNU there was a marked difference between the induction of 7-mG and the subsequent endpoints comet assay and micronucleus test. For example at 100 µM MMS or MNU the 7-mG adduct levels were 31 and 10-fold induced above background, whereas the respective increases in the comet assay and micronucleus test were only 3.1 and 1.7-fold and 2.5 and 1.6-fold (Table 4). For MMS this discrepancy increased further with dose due to the sigmoid increase of 7-mG. Mechanistically, methylation at nitrogen 7 of guanine is thought to destabilizes the N-glycosidic bonds between guanine and the 2′-deoxyribose and facilitate depurination to yield 7-methylguanine (7-mG) and an apurinic site [63]. Apurinic sites may hydrolyzed to DNA strand breaks under cellular pH conditions. However, DNA migration in the comet assay may not only be due to existing DNA strand breaks but also due to abasic sites, since these are converted into strand breaks under the pH conditions employed in the alkaline version of the assay (> pH 13). Since 7-mG adducts analyses and the comet assay were conducted at the same time after exposure an increase of the comet assay signal was expected equivalent (or proportional) to the increase in 7-mG. This was not the case. Relative increases in 7-mG levels were markedly greater for MMS and MNU when compared to changes in the comet assay or micronucleus test. Highly capable repair mechanisms must be active, reducing the high proportions of 7-mG to be converted to strand breaks or chromosomal damage. The discrepancy can further be explained by the fact that background 7-mG adducts constitute only a fraction of the background DNA damage observable with the unspecific genotoxicity endpoints comet assay or MN test. If only a specific fraction of background damage (7-mG) is increased, the total damage cannot show the similar increase (see also chapter 7, Fig. 33).

MMS was more potent than MNU for the formation of 7-mG and DNA strand breakage (comet assay and micronucleus test). The difference for 7-mG induction by MMS compared to MNU was 2.3, 3.1, 22, and 13-fold at 50, 100, 200 and 300 µM, the increases in the comet assay were 1.4, 1.8, 3.6, 5.4-fold, respectively. For micronuclei induction the difference between MNU and MMS was comparable to that in the comet assay (1.6, 2.5, 4.8-fold at 100, 200 and 300 µM, respectively). The higher potential of MMS to induce DNA strand breaks and chromosomal damage was reflected by the higher levels of 7-mG induction. However, quantitatively 7-mG did not serve as a good indicator to estimate the relative potential of the two agents for

DNA breakage. Up to 20-fold higher 7-mG levels induced by MMS compared to MNU were in contrast to 5-fold difference in potency to induce micronuclei. This means, the clastogenic potential of different alkylating agents may not be estimated based alone on the potential to induce 7-mG adducts. This further means that under the conditions of the assay 7-mG adducts were an inappropriate marker for DNA breakage and clastogenicity of MMS and MNU.

5.3.7.2 Induction of O^6-mdGuo versus mutant frequency

MMS clearly induced smaller increments to background of O^6-mdGuo adducts than MNU at equimolar concentrations (Table 4). In relative terms the increments of MMS were 6.3, 6.8 and 5.8-fold smaller at 50, 100 and 200 µM, respectively. Accordingly MNU was the more potent mutagen and induced more mutants (ca. 3-fold at all concentrations) when compared to MMS. In conclusion, the relative higher amounts of O^6-mdGuo adducts induced by MNU might explain the higher mutagenic potential of MNU, but the difference in magnitude (4 – 7 fold difference for O^6-mdGuo compared to about 3-fold for mutant frequency) between the two agents could not be explained based on O^6-mdGuo adducts alone. The higher amounts of 7-mG induced by MMS compared to MNU might also, besides being a clastogenic lesion, influence the mutant frequency, because 7-mG adducts also elicit mutagenic potential. If DNA polymerases pass by no instructive apurinic sites (possibly induced by 7-mG) they preferably incorporate adenosine (A-rule) [13,14]. If the original base was guanine this could lead to a G to A transition mutation. However, 20-fold greater increments in 7-mG induced by MMS compared to MNU show up in relative lower mutant frequency for MMS.

5.3.8 Extrapolation to low dose - low-effect region, doubling dose

For the comparison of the different endpoints, for dose-related increments above background in the low-effect region, the "concept of doubling dose" was used. The doubling dose is the concentration of agent necessary to double the background measure defined as 100%. Slopes of the linear low-dose part of the dose responses were derived from linear regressions using the minimum number of data (triplets at each concentration) necessary to get a significant slope >0. The slope was defined as the increase on the y-axis generated by a step of 1 µM on the x-axis. The relative

slope was calculated for each endpoint by dividing the slope by the respective background measure. Consequently, the relative slope provides a measure for increases relative to the background. The doubling doses for all endpoints were then derived from 1 divided by the respective relative slope (an increase in 100% represents a doubling of the background). The slopes and the resulting doubling doses for all endpoints are listed in Table 5.

About 5 – 15-fold higher MMS concentrations were necessary to double the background observed in the comet assay and the micronucleus test compared to the induction of 7-mG and O^6-mdGuo. For MNU the difference was even more pronounced and 10 – 100 fold higher doubling doses were necessary, respectively. Note that for the comet assay and the MN test all concentrations applied of MNU were required to achieve an significant slope, this increase was less obvious from the graph (Fig. 25).

Between the induction of DNA methylations and mutant frequency a discrepancy was observed, but to smaller extent as for adduct formation versus DNA breakage and chromosomal damage. The slope for O^6-mdGuo formation compared to the induction of mutant frequency was 2-fold and 4.5-fold steeper for MMS and MNU, respectively.

Table 5
Slopes of dose-response calculated by linear regression and resulting doubling dose for different genotoxicity endpoints assessed in L5178Y mouse lymphoma cells treated with MMS or MNU.

	slope[1] (no of data points[2])		relatives slope to BG[3]			doubling dose [µM]	
	MMS	MNU	MMS	MNU	Δ	MMS	MNU
7-mG	330 (2)	113 (2)	0.229	0.083	2.77	4.3	12
O^6-mdGuo	3.67 (4)	16.1 (2)	0.115	0.5	0.23	8.7	2
comet assay	0.0463 (2)	0.0444 (5)	0.022	0.005	4.4	45	200
MN	0.183 (3)	0.0778 (4)	0.015	0.0032	4.69	67	313
MF	2.97 (2)	8.23 (2)	0.046	0.11	0.42	21.7	9.1

[1] Expressed as the increase per µM agent.
[2] No. of data triplets (concentrations) required to achieve a slope by linear regression with $p < 0.05$
[3] Mean Background values: MMS series: 7-mG 1435; O^6-mdGuo 32; comet assay 2.1; MN 12; MF 64
MNU series: 7-mG 1349; O^6mdGuo 32; comet assay 8.77; MN 24; MF 78

In Fig. 29 the doubling-dose concept is illustrated graphically for MMS. Background levels of all endpoints were set to 100% and the linear low-dose parts of the five types of DNA damage induced by MMS are shown in the form of regression lines. The slope for DNA methylations was noticeably steeper when compared to the subsequent biochemical endpoints comet assay and micronucleus test or mutant frequency. The x-values at the intersections of the horizontal line (y = 200%) with the regression lines illustrate the larger MMS concentrations required to double the background measure of the genotoxicity endpoints comet assay and micronucleus frequency assay as compared to both types of DNA methylation.

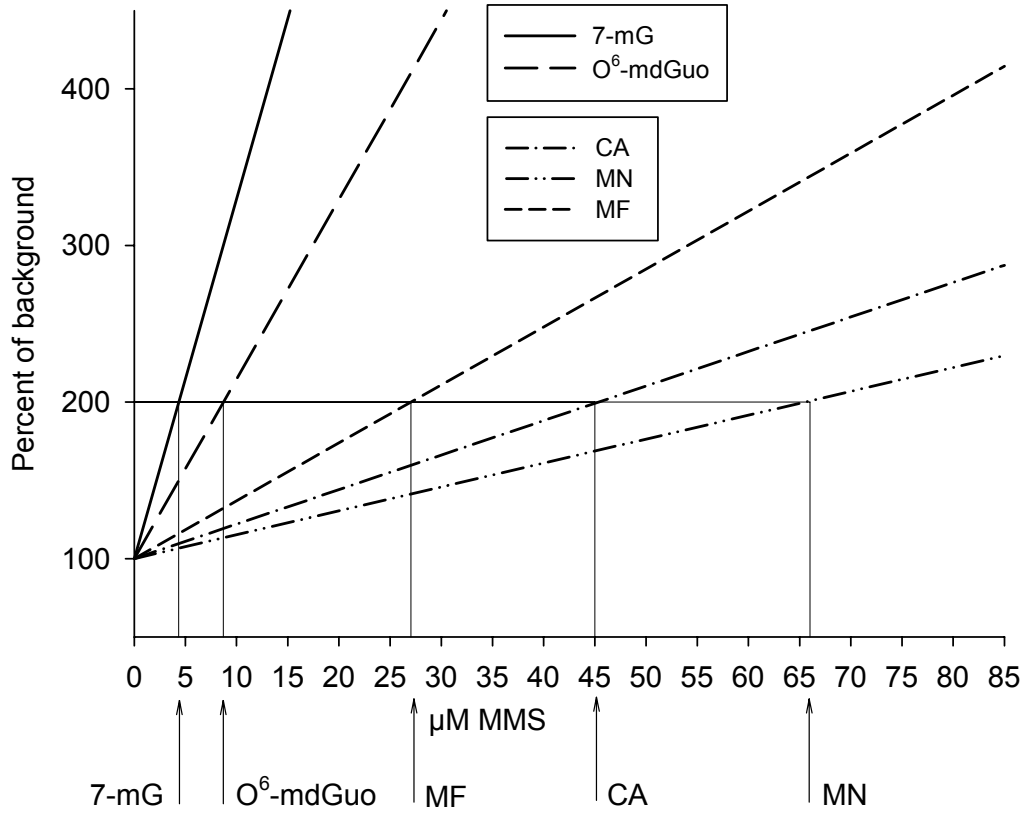


Fig. 29. Linear regression lines derived from the low-effect part of the dose response for 7-methylguanine (7-mG), O^6-methyl-2′-deoxyguanosine (O^6-mdGuo), comet assay analysis (CA), micronucleus test (MN) and $tk^{-/-}$ mutant frequency (MF) in L5178Y mouse lymphoma cells treated with methyl methanesulfonate (MMS). The values shown on the y-axis have been normalized to the control levels (0 µM MMS equivalent to 100%). The intersections of the horizontal line at 200% with the regression lines indicate on the x-axis the MMS concentrations required to double the background measures.

In a similar depiction for MNU (not shown) the discrepancy between the doubling doses between O^6-mdGuo (2 µM) and the comet assay (200 µM) and micronucleus test (313 µM) would be even more pronounced (compare Table 5).

5.3.9 Low-dose MNU: Experimental data

In the comet assay and the micronucleus test the effects of MNU were weak up to 300 µM (subsection 5.3.3), while O^6-mdGuo and mutant frequency induction were increased even the lowest concentration of 50 µM, followed by a linear dose response. In view of the fact that the induction of O^6-mdGuo was about 5-fold steeper than the induction of mutant frequency the question was addressed whether by decreasing the concentration of MNU a value could be reached, where DNA methylations are measurable, but the biological consequence "mutation" vanishes within the background fluctuation of the biological endpoint. Therefore, a series of experiments was added using smaller MNU concentrations. Again, the relative increases above background was calculated and the results for all endpoints are shown in Fig. 30. As expected, no increase above control values was observed for the comet assay or micronucleus test up to 50 µM MNU, whereas for 7-mG and O^6-mdGuo a linear increase above background was observed. For the mutant frequency the first concentration resulting in a significant increase above control was 20 µM MNU, followed by a linear increase with MNU concentration. In contrast, a 40-fold lower concentration (0.5 µM) resulted in a measurable increase of O^6-mdGuo above background values followed by a linear dose response (Fig. 30). Under the conditions of the assay the lowest observed effect level (LOEL) was 20 µM for the mutant frequency and 0.5 µM for O^6-mdGuo. However, this difference cannot be interpreted as a "threshold" for the induction of mutations because it also depends on the statistical power to detect an significant increase. Changes in dose spacing and sample number would result in different LOELs. The true shape of the dose-response curve for mutant frequency up to 20 µM is unknown and may include non-linear or even non-monotonic curves.

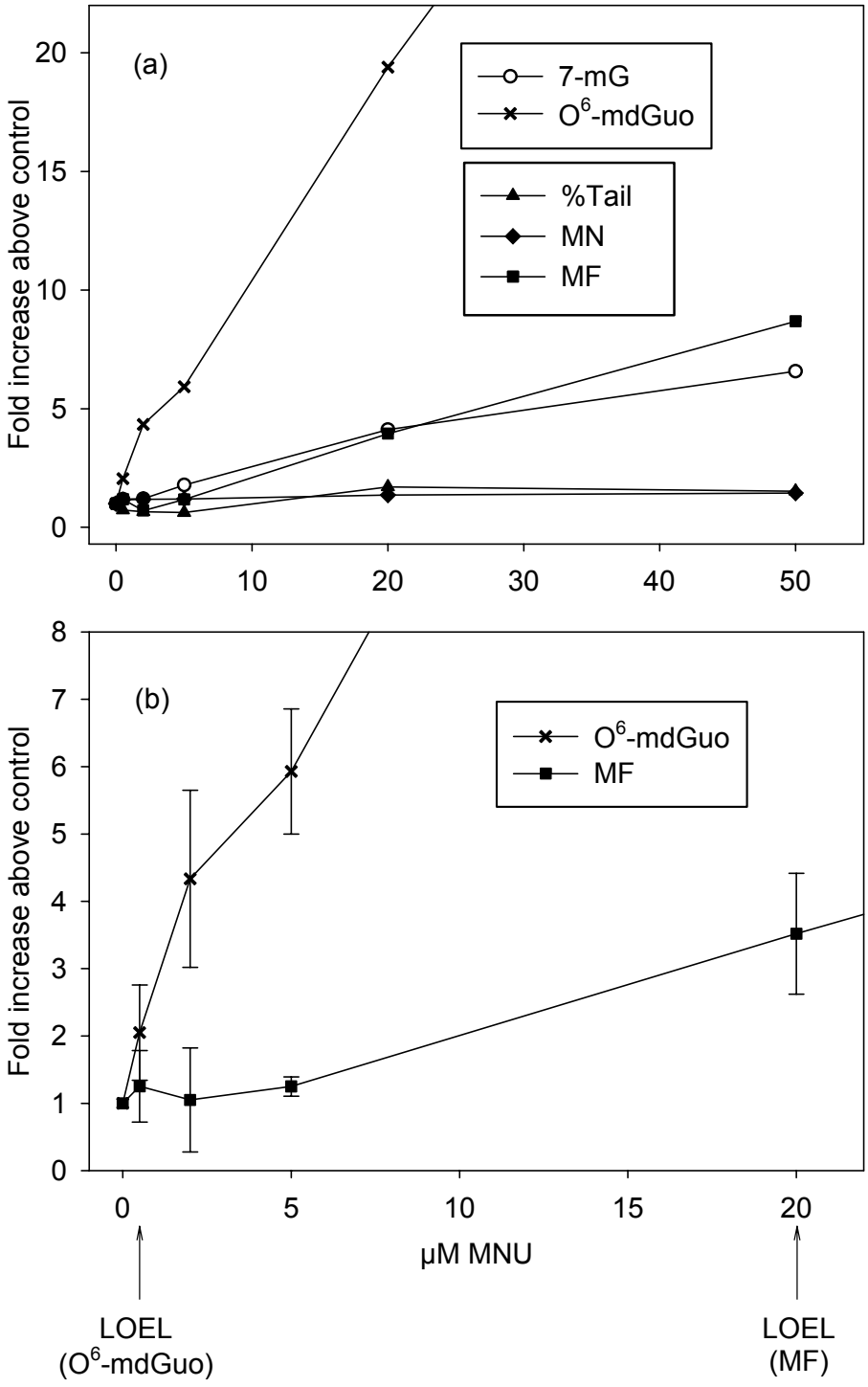


Fig. 30. (a) Comparison of relative differences (fold increase above control) for 7-methylguanine (7-mG), O^6-methyl-2′-deoxyguanosine (O^6-mdGuo), comet assay (CA), micronucleus test (MN), mutant frequency (MF) measured in L5178Y mouse lymphoma cells treated with N-methyl-N-nitrosourea (MNU) at different concentrations. (b) Zoom in for O^6-mdGuo and MF. The lowest observed effect level (LOEL) is indicated.

5.4 Discussion

5.4.1 DNA methylation and biological consequences

Large batches of L5178Y mouse lymphoma cells were treated with two different types of methylating agents. The increments of two types of DNA methylation (7-mG and O^6-mdGuo) and three endpoints of genotoxicity, (1) DNA strand breaks (comet assay), (2) chromosomal damage (micronucleus test) and (3) mutant frequency (mouse lymphoma thymidine kinase mutation assay) were evaluated concurrently. This parallel approach allowed for comparison of dose-dependent increments and relative slopes of the different endpoints.

The relative increase above background was much steeper for the DNA methylations than for the endpoints of DNA breakage and chromosomal damage to which these adducts may contribute. In quantitative terms, the MMS or MNU concentration necessary to double the biological endpoints DNA strand breaks and chromosomal damage was more than ten times higher (for MNU more than 100 times) than the concentration that results in a doubling of the "chemical endpoint" DNA methylation. With regard to biological significance a treatment related effect of alkylating agents could therefore be overestimated if it is based on increments in DNA adduct (methylations).

DNA methylations show a background prevalence possibly resulting from aberrant methylation of DNA by S-adenosyl-L-methionine (SAM), the physiological methyl group donor for the enzymatic methylation of cytosine to 5-methylcytosine [19]. SAM is present in various tissues at concentration of 25 to 50 µM [113]. There is strong evidence that aberrant background methylation of DNA is a phylogenetical old "threat", and cells have to counteract adequately. An exclusive repair mechanism for alkylations at O^6 of guanine exists, mediated by the *O*^{6}-alkylguanine-DNA alkyltransferase (AGT). AGTs have been identified in more than 100 different species over taxon boundaries including, Archaea, Bacteria, and Eukarya, sharing a highly conserved amino acid sequence surrounding the cysteine acceptor site [114]. AGTs transfer the alkyl group from *O*^{6}-alkylguanine to a cysteine residue and each molecule can act only once. The alkylated form of the protein is rapidly degraded by the ubiquitin/proteasomal system. For 7-mG adducts such a specific repair mechanism is not known and if compared to other background DNA adducts, 7-mG is present at relatively high background levels [115]. Accordingly 7-mG can be

measured relatively easy so 7-mG adducts are often used as a biomarker [116]. On the other hand one could conclude that 7-mG lesions might be the biologically less relevant lesion, when compared to O^6-mdGuo [117].

Methylation of DNA by electrophilic methylating agents like MMS or MNU have been shown at more than a dozen sites. (Note: the 5 position of cytosine is not a direct target for methylating agents). The 7(N) position of guanine is the predominately attacked nucleophilic site at the DNA bases and methylations at O^6 are formed at lower rate [11]. The ratio of the 7-mG to O^6-mdGuo depends on the chemical nature of the methylating moiety, as obviously shown here for MMS (S_N2-type) and MNU (S_N1-type). Literature values for the ratio of the rates of formation of 7-mG to O^6-mG is about 300 for MMS and between 6 to 14 for MNU [11,118]. This agrees well with the ratios seen in the linear low-dose range of our experiments at about 100 and 10 for MMS and MNU, respectively. The ratio for MMS was not constant over the entire dose range, possibly because of dose-dependent changes in the kinetics of depurination and/or repair of the two types of methylation. Observations in rat liver and kidney inferred that agents with greater amounts of oxygen alkylation showed greater carcinogenic effects [104]. Due to the high probability for mispairing with thymidine during DNA replication [119] these adducts are considered the biologically more important ones, although being not the quantitatively dominant lesions.

7-mG is assumed to contribute primarily to DNA strand breakage by facilitating depurination and subsequent hydrolysis of the apurinic site. Also intermediate (usually temporary) stages of base excision repair acting on 7-mG [14] result in DNA strand breaks. It was expected that the different levels of 7-mG induced by MMS and MNU will correlate with the respective biological endpoints of strand breakage measured here. Indeed MMS induced the comet assay signal and micronucleus frequency with steeper slopes, but these differences could not be explained on the basis of 7-mG alone. The increments observed in the comet assay and micronucleus test induced by MMS compared to MNU were smaller than they would have been estimated from the difference in 7-mG adducts alone. This means that other contributions to strand breakage induced by MNU (compared to MMS) cannot be excluded. One explanation might be that the miscoding lesion O^6-mdGuo possibly also give rise to double strand breaks during mismatch repair [102,103]. In general the data shown here do not prove that 7-mG or O^6-mGuo are causally related to the

measured biological endpoints. The observed slopes only indicate the dose-dependent increase in two specific adducts relative to background. Although unlikely, it cannot even be excluded that mechanisms other than DNA methylation are responsible for the MMS or MNU related increases in the biological endpoints. Investigations on all possible effects (including epigenetic effects) exerted by MMS or MNU are required for more information on this question.

A comparison of the induction of O^6-mdGuo adducts with the induction of mutant frequency was in particular indicated, because of the miscoding properties of this lesion. The question was, whether differences in the relative amount of O^6-methylation induced by the two methylating agents MMS and MNU correlate with their ability to increase the mutant frequency. MNU resulted in clearly steeper increases of O^6-mdGuo and mutant frequency indicating that O^6 is an appropriate marker for the mutagenic potency of MNU. However, if O^6-mdGuo would be the predominately mutagenic lesion responsible for the induction of gene mutations, differences in dose related increments between mutant frequency and O^6 between would be expected to be similar for MMS and MNU. But again a discrepancy was observed (difference in slopes between O^6 induction and mutant frequency was greater for MNU than for MMS) which could not be explained on the basis of O^6-mdGuo adducts alone. Mechanistically, it can be assumed that O^6-mdGuo adducts exert similar mispairing properties and are repaired similar by AGT, irrespective of the origin (MMS or MNU). But MNU induced higher levels as compared to MMS, therefore level-dependent differences in repair kinetics of the lesion cannot be excluded. However, in such a situation a deviation of linearity of the mutant frequency would be expected. 7-mG adducts also may give rise to mutations, indirectly mediated by apurinic sites. DNA polymerases tend to incorporate adenosine in opposite to a non instructive abasic site, thereby possibly generating base pair substitution mutations [13]. But, since the induction of 7-mG by MMS was larger compared to MNU and the corresponding mutant frequency of MMS was below that of MNU, the mutagenic potential of 7-mG cannot be entirely be excluded, but is unquestionably weak.

In conclusion the relative increase above background was much steeper for the methylations than for the endpoints to which these adducts contribute.

This indicates that DNA-adduct formation as a "first-line" product of the interaction of a genotoxic agent with its target may lead to a many-fold overestimation of the treatment-related biological relevance for more complex endpoints.

5.4.2 Choice of cell line and genotoxicity assays

Suspension cultures of the L5178Y mouse lymphoma cell line were used for two major reasons. First, they can easily be grown to large batches, which is necessary to obtain sufficient DNA for adduct analysis at low levels. Second, they are used in routine testing for different endpoints of genotoxicity [120] under standard guidelines and recommendations [37,40,77]. It should be noted here that the p53 gene product in L5178Y mouse lymphoma cells is dysfunctional due to a TGC (Cys) to CGC (Arg) transition in codon 170 in exon 5 [121]. This affects the response to DNA damage and influences DNA repair. The results shown here may therefore be specific for our experimental set-up and caution is advised for generalizations.

The comet assay, micronucleus test and mouse lymphoma assay were used as quantitative measures of DNA strand breaks, chromosomal damage and mutation, respectively. Note differences in the timeline of the assays: the comet assay (as well as the adduct analyses) reflects the situation at the end of the 4-hour incubation. The micronucleus test, on the other hand, adds in absence of the agent a period of 18 hours, which is necessary for nuclear division and expression of the DNA damage in the form of micronuclei. In the mouse lymphoma assay, an expression period of 48 hours is added. This means that DNA repair or modulation of the cell cycle may affect the results of the comet assay, the micronucleus test and the mouse lymphoma assay differently. It is interesting, therefore, that the relative slopes above background seen for the comet assay and micronucleus test were comparable, but more shallow compared to the increase of mutant frequency (Fig. 29).

5.4.3 Comparison of increments in different endpoints: doubling dose

The interpretation of the biological significance of chemically-induced DNA adducts depends on the quantitative relationship between induced increments of DNA adducts and increments of subsequent alterations on the cellular level being biologically relevant. To address this question DNA adduct levels induced by the methylating agents MMS and MNU were measured in parallel to subsequent

biological endpoints, to which these adducts may contribute. Both, the adducts and the biological endpoints showed background prevalence and where inducible by MMS and MNU. Therefore, induced increments in specific endpoints could be related to background. To calculate slopes above background for the different measures in the low dose region, linear regressions was employed in view of the fact that the rate of DNA adduct formation follows first order reactions, i.e., is proportional to concentration not only for diffusion and chemical reaction but also for enzymatic processes as long as the substrate concentrations are below the Michaelis constant. Aspects such as DNA repair, apoptosis or cell-cycle control (to name only a few) can modulate the outcome [122]. In particular for the loss (repair) of both 7-mG and O^6-mGuo first-order rates have been shown in DNA of rat liver and kidney a long time ago [123] and was confirmed recently [124] for O^6-mdGuo. Generally, shapes in the low dose part other than linear can never be excluded but the probability that the increase for DNA methylations could become more shallow than the slope for the biochemical endpoints is considered very low.

Several times the doubling dose of DNA methylations was required to double the biological endpoints. This discrepancy was marked for the induction of DNA methylations versus DNA breakage and, to lesser extent, for the induction of mutant frequency. This means that the contribution of increases in DNA methylations to increments in biological endpoints of genotoxicity may be overestimated. This infers that, if based on DNA adducts and considering the doubling of background measures of genotoxicity endpoints as an acceptable additional increment, considerably smaller concentrations of agent would be estimated being tolerable.

6 Biological significance of oxidative DNA damage

6.1 Kupffer cells and hepatocarcinogenesis

The major risk and etiology factors of hepatocellular carcinoma are well characterized [44]. They include infection with hepatitis B or C virus, chronic ethanol abuse and dietary exposure to mycotoxins (e.g. aflatoxin B1), as well as exposure to alkylating agents, such as nitrosamines, from dietary sources or from tobacco smoke. Hepatocellular carcinomas rarely develop in the absence of hepatitis, fibrosis and cirrhosis, which indicates that the inflammatory response might be a critical host factor. Inflammatory cells can generate an altered microenvironment that mediate detrimental effects on pathogens as well as on host cells. Kupffer cells are the macrophages of the liver involved in host defense and mediation of inflammation. Superoxide ($O_2^{\bullet -}$) is produced in Kupffer cells from oxygen (O_2) and NADPH catalyzed by the phagocyte NADPH oxidase (phox) according to the following reaction:

$$NADPH + 2\ O_2 \rightarrow NADP^+ + H^+ + 2\ O_2^{\bullet -}$$

Lack of the p47phox gene abolishes $O_2^{\bullet -}$ formation in phagocytes [46,47]. Superoxide is metabolized to various reactive oxygen species (ROS), which damage cells and DNA and enhance release of pro-inflammatory cytokines such as tumor necrosis factor alpha (TNFα) [25].

Superoxide generation from Kupffer cells contributed to hepatocarcinogenesis induced by diethylnitrosamine as has beenshown with p47phox knockout (*p47phox*$^{-/-}$) mice [47]. As a complete carcinogen diethylnitrosamine mediates tumorigenesis by activating both genotoxic and cytotoxic events. diethylnitrosamine mediates inflammation and cell death and subsequently triggers regenerative hyperplasia that may promote initiated cells to proliferate. This includes also the activation of Kupffer cells [47].

To examine whether *p47phox*$^{-/-}$ mice have reduced levels of oxidative DNA lesion and might be protected from hepatocarcinogenesis compared to their wild type (*wt*) counterparts we contracted a cooperation with the Institute of Cancer Research of the Medical University of Vienna. Carcinogenesis had been initiated in 6-week-old mice by a single dose of diethylnitrosamine followed by subsequent promotion by providing ethanol in the drinking water. Chronic ethanol shows mild tumor promoting effects [125] after exposure to genotoxins. This might be associated with the formation of oxidative DNA lesions. The mechanism by which ethanol induces oxidative stress is assumed to be based on the induction of CYP P450 2E1 but might also be mediated by stimulation of Kupffer Cells and via sustained inflammation. Cellular consequences of sustained oxidative stress and redox imbalance resulting from the combined actions of diethylnitrosamine and ethanol might result in enhanced formation of reactive oxygen species, lipid peroxidation and formation of oxidative DNA lesions.

The investigation of the combined effects of a nitrosamine and ethanol is particularly important because of the ubiquitous presence of nitrosamines and the fact that ethanol is widely consumed in beverages. The hypothesis was that differences in ROS formation between *wt* and *p47phox*$^{-/-}$ mice mediated through $O_2^{\bullet-}$ released by Kupffer Cells may be detectable by changes in the levels of background oxidative DNA lesions. This would show that ethanol might not only contribute to carcinogenesis via non-genotoxic tumor promotion but might also be genotoxic by indirectly altering levels of endogenously formed promutagenic DNA lesions. Background levels of two oxidative lesions 8-oxodGuo and εdAdo were measured simultaneously by LC-MS/MS in DNA from mice liver samples of *wt* and *p47phox*$^{-/-}$ mice treated with diethylnitrosamine, ethanol or in combination.

6.2 Oxidative DNA damage markers

Biomarkers for monitoring oxidative damage include the analysis of products from lipids (e.g. malondialdehyde and 4-hydroxynonenal), and from DNA (e.g. 8-oxodGuo, thymidine glycol, etheno or propano adducts) [29]. Here, two DNA adducts were examined, 8-oxodGuo and εdAdo (Fig. 6), formed by different mechanisms. 8-oxodGuo is formed by direct oxidation of DNA, while the exocyclic DNA adduct εdAdo is formed by the reaction of DNA with lipid peroxidation products [20]. Background levels of 8-oxodGuo have been determined in virtually all possible sources of DNA. This lesion is formed by a single oxidation step at the carbon in position 8 of guanine and artefact formation during sample work-up was described as critical. This may lead to overestimation but also to analytical variability of background 8-oxodGuo levels [69,70]. 8-oxodGuo was found to be a relatively weak mutagenic lesion inducing G to T mutations in bacterial and mammalian cells *in vitro* [126]. The pathway leading to εdAdo involves lipid peroxidation. The polyunsaturated fatty acids residues of phospholipids are sensitive to oxidation. Resulting lipid hydroperoxides can form reactive epoxides or aldehydes [65]. These reactive aldehydes can react to exocyclic DNA adducts like the promutagenic propano or etheno adducts [20,127].

It is doubtful that the moderately reactive superoxide (it also acts as an physiological signaling molecule [24]) is in fact an agent that directly attacks DNA to form 8-oxodGuo and it is generally assumed that conversion (via superoxide dismutase and H_2O_2) into the hydroxyl radical ($HO^{\bullet}$) is required [17]. The relevance of $HO^{\bullet}$, however, remains uncertain because it has an extremely short half-life, and, therefore has to be produced directly adjacent to DNA to induce 8-oxodGuo. The role of $O_2^{\bullet-}$ for lipid peroxidation and for the generation of εdAdo however is uncertain and may be different. The absence of $O_2^{\bullet-}$ production by Kupffer cells in *p47phox*$^{-/-}$ mice might therefore affect the levels of 8-oxodGuo and εdAdo differently.

6.3 Experimental

6.3.1 Mice

All mice were bred in the breeding facilities of the Decentralized Biomedical Facility of the Medical University of Vienna (Himberg, Austria). Wild type (*wt*) B6,129 and *p47phox*$^{-/-}$ mice had been provided by Dr. Steven M. Holland, Laboratory of Host Defences, National Institutes of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD. *p47phox*$^{-/-}$ mice were originally produced from embryonic stem cell 129/Sv-derived sperm and C57BL/6-derived eggs. Mice were obtained by caesarean section and maintained under specific pathogen-free (SPF) barrier conditions until termination. They were adapted for at least 1 week under standardized SPF conditions (Macrolon cages, 2 – 3 mice per cage, 20 ± 2°C room temperature, 50 – 60% relative humidity, and artificial lighting with lights on from 6 a.m. to 6 p.m.). Sterilized laboratory chow and water were provided *ad libitum*.

6.3.2 Treatment

Six week old (20 g) male mice were treated once with diethylnitrosamine (DENA), 90 mg/kg intraperitoneal (i.p.) in PBS or with PBS alone. Five animals per group were treated, except for the groups "*wt*/untreated (n = 4)" and "*p47phox*$^{-/-}$/DENA (n = 6)". After a recovery period of 3 weeks mice were exposed to ethanol in drinking water. The ethanol concentration was increased in 3 weekly steps from 3% to 5% to 8% (g/g). The animals were killed after 35 weeks by asphyxiation in carbon dioxide. The livers were excised, blotted, weighed and samples were immediately frozen in a mixture of isopentane/liquid nitrogen and stored at -70°C until analysis.

6.3.3 DNA isolation, hydrolysis and LC-MS/MS analysis

Coded aliquots of liver of *p47phox*$^{-/-}$ and *wt* mice were received on dry ice from the Institute of Cancer Research, Vienna, Austria (Dr. H. Schulte-Hermann and Dr. W. Parzefall). Genomic DNA was isolated from approximately 200 - 300 mg of liver using a DNA isolation kit (Nucleobond® AXG, Macherey-Nagel, Düren, Germany) following the manufactures' instruction as described before (subsection 3.4.7). The approximate yield of genomic DNA was on the order of 100 µg DNA per 100 mg

tissue. The DNA pellets were dissolved in water and hydrolyzed using nuclease P1 and alkaline phosphatase as described in subsection 3.4.7. To quantify 8-oxodGuo and εdAdo the samples were analyzed by LC-MS/MS as described in detail in subsection 3.4.3 with the following modifications. Per sample, 400 µl of hydolysate containing 820 nmols of normal nucleotides were injected. The backflush flow rate was 400 mL/min instead of 500 mL/min. This resulted in slightly longer retention times (chromatogram not shown) but increased the sensitivity for 8-oxo-dGuo and εdAdo (data not shown) possibly because of improved evaporation efficiency in the electrospray source.

6.3.4 Data analysis

For graphical representation and exploratory analyses the levels of 8-oxodGuo and εdAdo were expressed as adducts per 10^9 normal nucleotides. Prior to statistical analysis the data were $\log_{10}$ transformed to decrease deviation from a normal distribution and enable analysis of variance (ANOVA). In the first series of data three experimental factors were varied. (1) Wild type or *p47phox*$^{-/-}$ mice, (2) diethylnitrosamine (yes or no), and (3) ethanol (yes or no). Therefore, a three-way analysis of variance was conducted to test for differences and interaction between the factors. Interaction would indicate interference of factors. If a statistically significant interaction between factors was observed, differences between groups were analyzed using Student′s t-test, followed by pairwise comparison of treatments, separately for *wt* and *p47phox*$^{-/-}$ mice using Tukey′s HSD test. A significance level of $p = 0.05$ was used. In the second series of experiments (*wt* vs. *p47phox*$^{-/-}$; combination treatment vs. no treatment) a two-way ANOVA was carried out. Differences in adduct levels were expressed in multiplicative terms. All statistical analyses were performed by Dr. Roman W. Lutz (Seminar for Statistics, ETH Zürich, Switzerland) using the free statistics software "R" (http://www.r-project.org).

6.4 Results

Fig. 31a shows levels of 8-oxodGuo adducts measured in DNA from mice liver from the first experimental series. In untreated *p47phox*$^{-/-}$ mice a trend towards lower 8-oxodGuo levels was observed, when compared to *wt* mice (1.2-fold, p = 0.08).

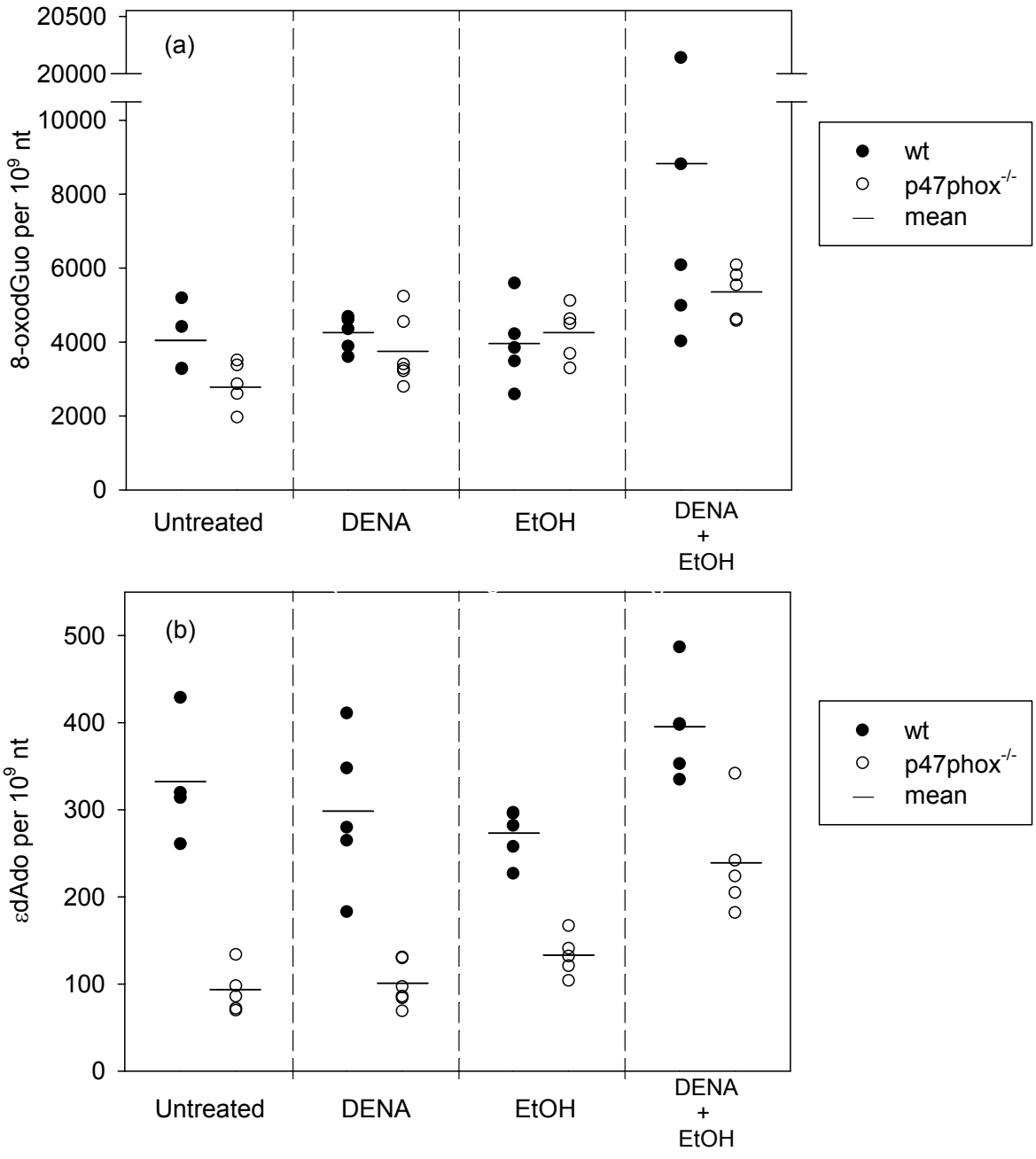


Fig. 31. Analysis of oxidative DNA adducts in wild type (*wt*) and *p47phox*$^{-/-}$ mice. Levels of (a) 8-oxodGuo and (b) εdAdo were determined in DNA isolated from liver of untreated mice and after treatment with diethylnitrosamine (DENA) or ethanol (EtOH), and in combination. Adduct levels are given as adducts per 10^9 normal nucleotides (nt).

This difference was no longer apparent after treatment with diethylnitrosamine or ethanol, but was clearly observable after the combination treatment with the two agents. Within the *p47phox*$^{-/-}$ and *wt* mice groups the separate treatments alone did not result in increases of 8-oxodGuo, but the combination treatment clearly increased 8-oxodGuo levels (2-fold, $p < 0.002$) compared to control mice. The levels of εdAdo (Fig. 31b) were clearly reduced in *p47phox*$^{-/-}$ mice compared to their *wt* counterparts in all four treatment groups (2.5-fold, $p<0.00001$, t-test, $t = 8.1$, $n = 20$). Further, the combination treatment with diethylnitrosamine followed by chronic ethanol resulted in a significant increase of εdAdo levels in *p47phox*$^{-/-}$ mice compared to untreated mice (2.6-fold, $p = 0.00004$). In their *wt* counterparts this effect could not be demonstrated significantly. In *p47phox*$^{-/-}$ mice the combined treatment resulted not only in increased levels of εdAdo when compared to untreated controls, but also when compared to the isolated treatments with diethylnitrosamine (2.4-fold, $p = 0.00006$) and ethanol (1.8-fold, $p = 0.0067$) alone. In *wt* mice the combined treatment diethylnitrosamine followed by ethanol showed a trend to increased levels of εdAdo (1.4-fold, $p = 0.056$), but not when compared to the untreated control but in comparison to ethanol treatment alone. Since ethanol applied alone resulted in a slight (not significant) reduction in εdAdo compared to the untreated control this result should be interpreted with caution.

In summary a reduction of oxidative lesions in *p47phox*$^{-/-}$ mice could be clearly demonstrated for εdAdo, but for 8-oxodGuo the decrease was only mild (and with $p = 0.077$ close at the significance limit of $p \leq 0.05$). The combination treatment of diethylnitrosamine and ethanol resulted in increased levels of 8-oxodGuo in both *p47phox*$^{-/-}$ and *wt* mice. For εdAdo this effect could only be shown for the *p47phox*$^{-/-}$ mice. To further corroborate the hypothesis that deficiency in superoxide generation by Kupffer cells in *p47phox*$^{-/-}$ mice might result in reduced levels of oxidative lesions in liver DNA, a second series of *p47phox*$^{-/-}$ and *wt* mice liver was analyzed for 8-oxodGuo and εdAdo.

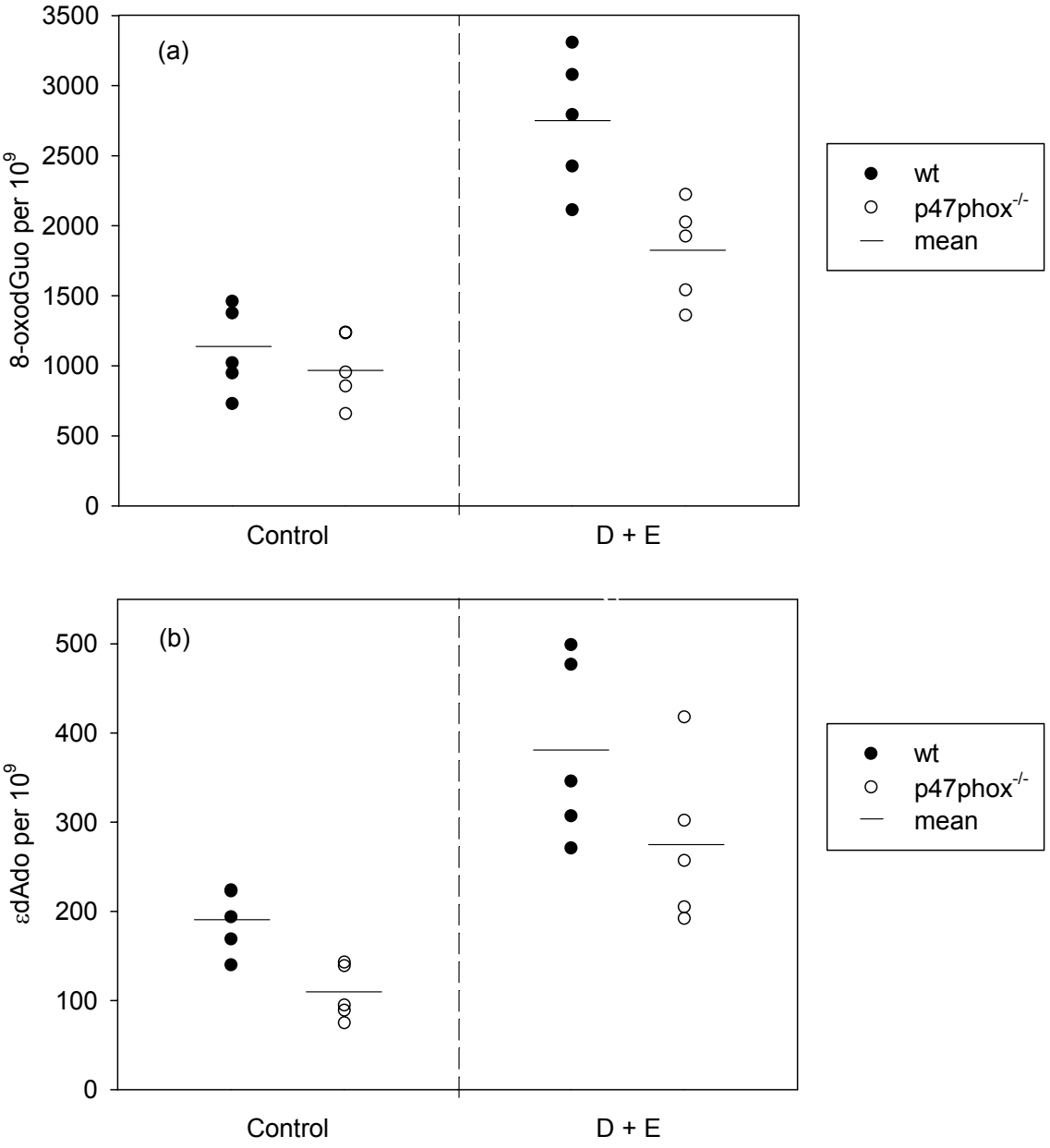


Fig. 32. Analysis of (a) 8-oxodGuo and (b) εdAdo in mice liver. Wild type (*wt*) and p47phox knockout (*p47phox*$^{-/-}$) mice were treated with diethylnitrosamine (DENA) followed by ethanol (EtOH). DNA adduct levels are given as adducts per 10^9 normal nucleotides.

The levels of 8-oxodGuo and εdAdo measured by LC-MS/MS in the second experimental series are shown in Fig. 32. For both adducts 8-oxodGuo and εdAdo lower levels were found in *p47phox*$^{-/-}$ mice than in their *wt* counterparts. Hence, the observation from the first series (Fig. **31**) that *p47phox*$^{-/-}$ reduces levels of promutagenic oxidative DNA adducts in mice was confirmed. The levels of 8-oxo were 1.3-fold reduced ($p = 0.028$) and of εdAdo 1.5-fold ($p < 0.004$) (Two way

ANOVA, no significant interactions). Like in the first series the effects of mice type on 8-oxodGuo was smaller compared to the effect on εdAdo levels. Further, the second series again showed that the combination treatment with diethylnitrosamine followed by ethanol resulted in an increase in 8-oxodGuo (2.2-fold, $p < 0.0001$) and in εdAdo lesions (2.2-fold, $p < 0.0001$) in both *wt* and *p47phox*$^{-/-}$ mice.

6.5 Discussion

Mice deficient in phagocyte NADPH oxidase (*p47phox*$^{-/-}$ mice) are less sensitive to hepatocarcinogenesis induced by diethylnitrosamine [47]. To test whether the abolished release of superoxide from Kupffer cells also results in reduced levels of oxidative DNA damage, the LC-MS/MS method developed for the determination of background DNA adducts (section 3) was applied to DNA isolated from liver of *p47phox*$^{-/-}$ and *wt* mice. Lower levels of the promutagenic DNA adducts 8-oxodGuo and εdAdo were observed in *p47phox*$^{-/-}$ mice compared to *wt*. DNA adduct formation mediated by superoxide released by Kupffer cells might therefore be a contributing host factor in chemically-induced hepatocarcinogenesis.

The two DNA adducts examined, 8-oxodGuo and εdAdo, are formed by different mechanisms. 8-oxodGuo is formed by direct oxidation of DNA while εdAdo is formed by the reaction of DNA with lipid peroxidation products [20]. Since the decrease observed for εdAdo in *p47phox*$^{-/-}$ mice compared to *wt* was greater than for 8-oxodGuo this infers that superoxide might rather be involved in lipid peroxidation than in direct interaction with DNA. The phagocyte NADPH oxidase in Kupffer cells transfers electrons from NADPH from the cytosolic side across the membrane and couples these to molecular oxygen to produce superoxide. Thus, the release of superoxide occurs close to polyunsaturated fatty acids residues of phospholipids of membranes. Possible products resulting from lipid peroxidation might be reactive electrophilic agents like malondialdehyde or 4-hydroxy-2-nonenal and are potential intermediates to generate different exocyclic adducts to DNA, such as εdAdo [128,129]. The exact mechanism of εdAdo formation is not known, but enhanced formation of lipid peroxidation products was linked to enhanced formation of εdAdo [129].

Significant increases in oxidative DNA adducts in both *wt* and *p47phox*$^{-/-}$ mice were observed for the combination of diethylnitrosamine initiation followed by

repetitive ethanol exposure. For the isolated treatments with diethylnitrosamine or ethanol alone, such an effect was not observed. Within the mice types, neither the single dose of diethylnitrosamine nor the chronic ethanol treatment resulted in significantly increased levels of oxidative lesions after 35 weeks.

Diethylnitrosamine induces ethylations in DNA, including the highly promutagenic O^6-adducts of guanine [118]. Further, diethylnitrosamine directly or indirectly stimulates Kupffer cells to release proinflammatory agents like TNFα, superoxide and other cytokines [47]. Since our analysis of DNA was performed after a long recovery period (35 weeks) any early damage will have been repaired. Ethanol was provided chronically but no increase of oxidative lesions was seen either when without DENA initiation. This finding supports the assumption that ethanol alone is neither directly genotoxic, mutagenic nor clastogenic [130,131] but might enhance cytotoxicity induced by alkylating agents [132]. The conditions of the combination treatment, however, were different. In the combination the initial inflammatory host response induced by diethylnitrosamine was possibly sustained by the chronic ethanol treatment. In this situation, ethanol might act as a tumor promoting agent that sustains a pro-oxidative effect initiated by diethylnitrosamine. This emphasizes that the terms tumor promoter and non-genotoxic carcinogen are descriptive and should not be used as synonyms. Under certain circumstances agents like ethanol might elicit (indirectly) both tumor promoting and genotoxic effects simultaneously, contributing possibly to tumor initiation, promotion and progression.

Although *p47phox*$^{-/-}$ mice showed reduced levels of the two adducts, a background was measurable in all untreated mice including *p47phox*$^{-/-}$. This indicates (i) that superoxide released by Kupffer cells in the *wt* mice contributed to the background of oxidative DNA lesions and (ii) that phox-unrelated sources of oxidative DNA damage exist in the liver. Since the differences in 8-oxodGuo levels between *wt* and *p47phox*$^{-/-}$ mice were only small (1.2 and 1.3 fold) it might even be concluded that for the background of 8-oxodGuo in homogenized liver tissue the role of superoxide released from Kupffer cells is of minor importance.

Oxygen radicals attack many cellular components, not just DNA. From a quantitative standpoint, e.g. in comparison to glutathione, unsaturated fatty acids, or proteins, the nuclear DNA might be a target of minor importance [20]. However, the damage to other cellular macromolecules than DNA, induced by oxygen radicals might also be important for tumorigenesis. The alteration of structure and function of

cancer-related proteins by oxidative stress may affect cell growth and tumor promotion by activating signal transduction pathways. This can result in the induction of proto-oncogene as has been shown e.g. for c-FOS, c-JUN or c-MYC [133] which may in turn affect DNA repair, cell cycle checkpoints and cell death and mediate the carcinogenesis of chemicals.

7 Discussion

7.1 Analysis of DNA adducts by LC-MS/MS

A large number of chemical carcinogens can bind covalently to DNA (*per se*, or via chemically reactive intermediates) resulting in a variety of DNA adducts. Different methods have been developed for the detection, identification and quantification of DNA adducts. Comprehensive reviews discussing the merits and limitations of the different approaches exist [134,135]. Most commonly used methods due to high sensitivity are based on ^{32}P postlabeling. Under optimum conditions, these methods provide sensitivity down to 1 adduct per 10^9 but specificity and reproducibility is poor. Mass spectrometry coupled to liquid chromatography (LC-MS/MS) is to date not as sensitive, but highly specific [54]. Further LC-MS/MS allows to quantify more accurately through the use of stable isotope labeled internal standards.

In this work LC-MS/MS methodology was developed for the analysis of the DNA adducts 7-methylguanine, (7-mG), O^6-methyl-2′-deoxyguanosine (O^6-mdGuo), 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxodGuo), and 1,N^6-etheno-2′-deoxyadenosine (εdAdo) and was successfully applied to determine background and induced levels in DNA of various sources (chapter 3: rat liver, chapter 5: cultured cells and chapter 6: mouse liver). For O^6-mdGuo, 8-oxodGuo and εdAdo, the method was developed

based on the 2′-deoxyribonucleosides. The stable isotope labeled O^6-[$^2H_3$]mdGuo was synthesized and used as an internal standard, to achieve acceptable accuracy and reproducibility. For 7-mG the method was based on the base adduct, since the respective 2′-deoxynucleoside is unstable and readily depurinates [64]. Analyzing the 2′-deoxynucleosides ascertains that the adducts under study originate from DNA and not from RNA. Further, all 2′-deoxynucleosides show a sensitive transition in the multiple reaction monitoring mode i.e. the neutral loss of 116 amu (cleavage of the β-glycosidic bond) [30].

Sample work-up is considered to be a critical step in the quantification of DNA adducts by LC-MS/MS [135]. Low levels of DNA adducts are embedded in a matrix in which there could be up to 10^7 to 10^8-fold excess of unmodified bases. Each additional step, such as sample transfer, dry-down steps or solvent exchange during work-up increases the probability of sample loss. Furthermore, the possibility for introduction of artifacts increases as the sample processing becomes more complex, in particular if only a single oxidation step is required, like for the formation of 8-oxodGuo [69]. The reduction of sample work-up steps was achieved in the present work by employing an on-line sample preparation using a column switching approach. These approaches were initially developed for high-throughput analysis of pharmaceutical compounds and their metabolites by LC-MS/MS in complex matrices like plasma or urine [55]. Recently, on-line sample clean-up techniques employing column switching have been used in DNA adduct analysis [53,136] and have great promise for future applications.

Most chemical carcinogens form a number of different DNA adducts and it is advised to determine different adducts within the same analytical run. Here we focused on a multi-adduct method for O^6-mdGuo, 8-oxodGuo and εdAdo, three adducts formed endogenously and exogenously by DNA methylation, direct oxidation of DNA and reaction of DNA with lipid peroxidation products, respectively. For the single adducts such approaches do not provide the highest possible in terms of LOD, but represents a compromise between sensitivity and flexibility. Apparently, the development of multi-adduct methods is becoming a common approach for the study of the effects of chemical carcinogens, as very recently shown for adducts from alkylating agents [49,53] or for various adducts caused by oxidation or lipid peroxidation [137,138].

A screening approach for DNA adducts with unknown structures, based on their shared constant neutral loss (CNL) of 116 amu (2′-deoxyribose) that all nucleosides have in common, was recently developed [139]. This approach (also named "adductomics" following other "omics" approaches) aims to screen for differences between groups or treatments based on a large number of background or exposure-related DNA adducts. Such an approach may give further insights in the formation, removal and significance of DNA adducts induced by chemical carcinogens. Since this screening is based on the loss of the 2′-deoxyribose as a neutral moiety, it screens for all 2′-deoxyribonucleosides (know and unknown) which might then be structurally characterized in the same analytical run by the acquisition of product ion spectra. The instrument of choice for such "information dependent acquisition experiments" (CNL combined with the acquisition of product ion mass spectra) is a combination of a tandem mass spectrometer with a sensitive ion trap in a single instrument. However to realize such experiments at low background levels the LC-MS/MS methodology in terms of sensitivity require another large step forward. Only then will the respective analyses become the "gold standard" in DNA adduct determination [30,35].

7.2 Comet assay analysis

The comet assay has been extensively used in genotoxicity testing [140], human biomonitoring [141], as well as in fundamental research in DNA damage and repair [142-146]. This fast and easy method to estimate DNA damage in single eukaryotic cells is based on migration of DNA fragments during electrophoresis away from undamaged DNA [147]. Because DNA fragmentation that may occur secondary to chemically-induced cytotoxicity (e.g. mediated by endogenous nucleases) the comet assay, may be subject to potential artifacts by non-genotoxic cell death. Genotoxicity is only biologically relevant if it occurs in cells capable of surviving the damage. Therefore, the assessment of cytotoxicity is of key importance [79]. The comet assay has achieved the status of a standard test in the battery of tests used to assess the safety of novel pharmaceuticals or other chemicals [76]. The implication of comet assay results for hazard identification and risk assessment is currently under discussion. In accordance with international guidelines for genotoxicity testing the *in vivo* comet assay is already recommended for follow-up testing of positive *in vitro*

findings in particular for organs or cell types which cannot easily be evaluated with other standard tests [140]. Results from the comet assay will therefore, have practical consequences in the risk assessment processes and further development of substances.

This emphasizes the importance of appropriate acceptance criteria for assessment of cytotoxicity in the assay. A lower limit for cytotoxicity appeared to be at 75% of viable cells at the time point of comet analysis as suggested by Henderson et al. [93] but this estimation does no take into account the different qualities of cell viability assays. It appears reasonable to set different cytotoxicity limits depending on the quality of method used for cell viability testing. Methods based on dye exclusion by membranes require probably more stringent limits than e.g. specific markers of early apoptosis or the assessment of long-term survival.

In the present work, the comet assay was used as a quantitative marker for DNA breakage, alkali-labile sites as a consequence of DNA methylation for dose-response analyses. Over the entire range of concentration used the viability assessed by annexin V/propidium iodide double staining was greater than 80%. However, it cannot be excluded that cytotoxic effects even within this range of 20% may influence the results, regarding the shape of dose response. This might hold true not only for the comet assay, but also for any other *in vitro* genotoxicity test system.

7.3 Biological significance of DNA adducts

7.3.1 Relating increments of DNA adducts to biological consequences

A large number of endogenous DNA damaging agents and processes constantly damage DNA and contribute to a background level of DNA damage [17]. Exposure to an exogenous carcinogen gives rise to an incremental DNA damage. At low dose levels the additional damage is expected to be proportional to dose, as long as all enzymatic processes for metabolic activation and detoxication are below the concentration equivalent to the Michaelis constant and follow first order kinetics.

On this basis, there cannot be any low dose that would not be associated with some – perhaps negligible – risk. In view of the background DNA damage resulting from endogenous and unavoidable agents and actions it appears reasonable to think in terms of increments above background for the contribution of DNA adducts resulting from exposure to exogenous carcinogens to the spontaneous process of carcinogenesis. In this work this question was addressed *in vitro* using large batches of L5178Y mouse lymphoma cells treated with MMS and MNU. These methylating agents showed a different profile of 7(N) versus O^6 methylation of guanine. The formation of 7-mG and O^6-mdGuo was determined and in parallel, using the same population of cells, three different genotoxicity assays were performed.

The adducts 7-mG and O^6-mdGuo are both also endogenously formed background lesions, MMS and MNU therefore produce additional DNA methylations to an already existing background. This increment could be expressed as "fold" induction to background and it was possible to estimate the dose of the methylation agents that doubled the background ("doubling dose" concept). If expressing increments in multiplicative terms (fold induction above background) the size of the background measurement is of major importance and is a highly decisive factor in this type of analysis. It is therefore important to emphasize two points. First, that background DNA damage represents a steady-state level between formation and removal by repair and that background measured by the different genotoxicity endpoints reflect different qualities of background DNA damage.

DNA adducts are specific primary DNA lesions and usually contain information on the genotoxic agent involved (here: methyl-group). The subsequent biochemical and biological endpoints comet assay, micronucleus test and the mouse lymphoma

thymidine kinase mutation assay are different. Parameters observable e.g. in the comet assay, no longer allow to retrace the responsible DNA reactive agent or process. The biological endpoints embrace various (and probable numerous) individual contributions of specific DNA lesions, which finally result in the observable effect of the biological endpoint. Thus, the formation of 7-mG or O^6-mdGuo due to MMS or MNU exposure is only a fraction within other lesions that in total generate the outcome in the biological endpoints. This explains why a doubling of specific adducts, that only represent a part of the observed background damage, cannot result in a doubling of the biological consequences (Fig. 33).

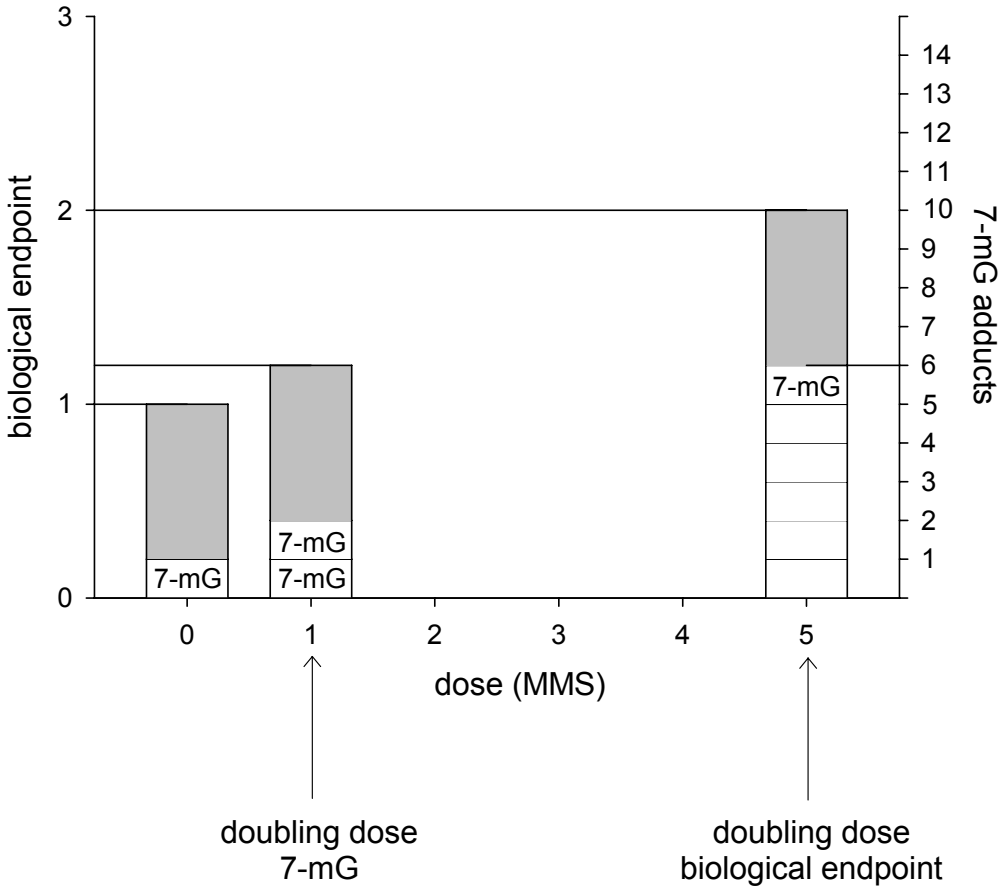


Fig. 33. Relative increases of a specific DNA adduct (7-mG) with background prevalence and the resulting increases in the biological consequence (e.g. DNA strand breaks) to which the specific adduct contributes. At dose zero the background of the specific adduct (white part of the bar, right y-axis) contributes to the total background observed in the biological endpoint (left y-axis). A specific doubling of adducts at dose 1 cannot result in a doubling of the total background. At the doubling dose of the unspecific endpoint in this example a 6-fold induction of the specific adduct was required.

The relative contribution of the measured type of DNA damage (7-mG in this example) as a fraction of the total damage (shaded plus unshaded parts of the bar)

depends not only on the dose but also on importance of the sum of all types of damage other than the measured one. For illustration purposes 7-mG was chosen as an example. In fact 7-mG is not the only lesion induced by MMS or MNU. However, the conclusions will also hold true when all effects induced by MMS and MNU that contribute to the unspecific biological measure would be represented by the white part of the bar. The grey part would then be smaller but never zero (there will always be parts of background observable in the biological endpoint, which are not related to MMS or MNU, e.g. chemical instability of DNA, error rate of DNA polymerase). A doubling in all MMS-specific lesions therefore cannot result in a doubling of the biological endpoint. This fact has to be kept in mind if data on DNA adducts are proposed to be included into safety assessment of chemicals and for the contribution of DNA adducts to biologically relevant endpoints.

Background values measured in biological endpoints can be influenced in many ways. For the thymidine kinase assay, the L5178Y mouse lymphoma cell cultures had been cleansed by pretreatment with methotrexate in order to reduce the number of spontaneous mutants $tk^{-/-}$ in the culture. However, the background mutant frequency could not be reduced to zero. This rises two questions, (i) why were not all background mutants killed by the cleansing and (ii) what does the background in mutant frequency represent after cleansing (selection of only resistant mutants). Cleansing is inhibition of the *de novo* synthesis (inhibition of dihydrofolate reductase) of thymidine to be incorporated into newly synthesized DNA. This inhibition is dose dependent and might not be 100% at the concentration of inhibitor used. Therefore, a certain number of cells might survive even without a functional thymidine kinase copy. The total background therefore represents the sum of pre-existing mutants that survived the cleansing and mutants that were induced in the period between end of cleansing and treatment (*de novo*-mutants). The difference cannot be resolved using the standard fluctuation assay because the origin of the colonies cannot be traced back. The selective agent TFT induces growth arrest in $tk^{+/-}$ cells and selects *de novo* induced as well as pre-existing mutants. The number of *de novo* mutants is smaller than the sum of de novo and pre existing mutants, therefore calculated increments to background would vary depending on the mutant fraction aimed to be used. Since the mouse lymphoma thymidine kinase mutation assay is part of the standard battery for genotoxicity testing of chemicals and is the preferred mammalian cell mutation test in many regulatory documents [148], an approach to distinguish between the *de*

novo induction of thymidine kinase mutants from the selection of pre-existing mutants remains an issue to be addressed if chemically-induced increments are to be related to background mutant frequency.

7.3.2 Dose response for DNA adducts without background

For DNA-reactive xenobiotics that form adducts that are not present as background, untreated controls show zero adducts so that the data cannot be expressed in percent of background. In the absence of background, the dose-response relationship starts at point (0,0) as shown in Fig. 34 (solid line). This might be the case for e.g. cytostatic drugs or pesticides. A limit of detection (LOD) for xenobiotic adducts is also indicated, together with the corresponding lowest observable effect level (LOEL) on the dose axis. In this situation of zero background, the LOD for adducts solely depends on the assay sensitivity, and all methodical improvement in adduct detection results in a reduction of the LOD and the corresponding LOEL.

The situation is different for the biological endpoint of genotoxicity shown by the interrupted line originating from the background measure (Fig. 34). For a significant increase above background, the so-called critical level of the difference between treated and controls has to be reached. The critical level is associated with the statistical test used for comparing the treated group with a control group. It depends on the variance of both, treated and control group. It is therefore higher than the LOD for a measure with zero background, and any reduction of the critical level – and with this of the LOEL – by methodical improvement is limited by the variance in the background (control level).

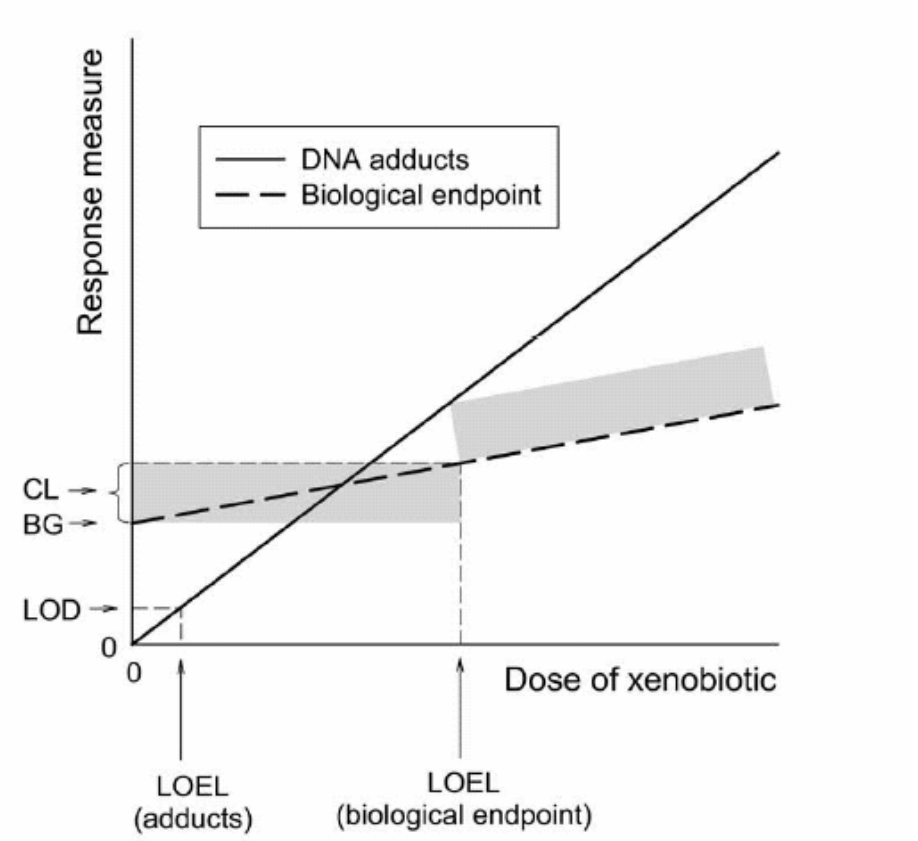


Fig. 34. Schematic representation of the dose response for a DNA adduct formed by a xenobiotic (zero background in controls) and a biological endpoint of genotoxicity (with measurable background). Proportionality to dose was assumed for both types of endpoints. The limit of detection (LOD) for adducts corresponds to the lowest observable effect level LOEL on the dose axis. For the biological endpoint, the critical level (CL) represents the minimum size of the increase that is required to achieve statistical difference to controls. This level defines the LOEL for the biological endpoint.

This difference between xenobiotic adducts and biological endpoints of genotoxicity with respect to the LOEL has an impact on the appearance of the respective dose-response curves. The span between the LOEL for adduct formation and the LOEL for biological endpoints with background widens with each increase in sensitivity for adduct detection. Adducts may show a statistically significant increase in a dose range that may be far below the dose required to produce a significant increase in a biological endpoint. This range is shown by the shaded area for the biological endpoint as a horizontal rectangle. Only above the LOEL the difference to control is statistically significant. Since the LOEL may appear as a breakpoint in the dose response between slope 0 and slope >0, a threshold is more readily postulated for a

biological endpoint than for xenobiotic adduct formation. Since this is based on statistical grounds only, it should not be over-interpreted [149].

7.3.3 Tolerable increment in DNA damage

A number of approaches can be discussed to define tolerable exposure levels to chemicals that form DNA adducts. The first is based on the concept of the doubling dose introduced with Fig. 29. A tolerable dose could be defined as a fraction of the dose that results in a doubling of the background. The size of this fraction could depend on the exposure scenario. Exposures of the entire population would have to be handled more stringently than exposures at the workplace. Another point should also be considered: Doubling doses do not tell us how effectively a given background measure may lead to clinical manifestations. If the background contributes a substantial part to the total "spontaneous" cancer risk, the tolerable fraction would have to be small. This holds, for instance, for ionizing radiation and radioisotopes where average background exposure may be responsible for a lifetime cancer mortality in the low percent range.

A second approach could be based on differences between individuals or day-to-day variability within individuals. Exposure-related increments that do not exceed some fraction of the standard deviation in a population or fluctuation with time could be considered tolerable. The third approach entails comparison of increments with effects from largely unavoidable or generally accepted risk factors. Heat-related formation of DNA-adduct forming carcinogens such as aromatic amines, polycyclic aromatic hydrocarbons, and acrylamide are an example. On this basis, adducts resulting from exposure to a xenobiotic food contaminant could be limited to a fraction of the unavoidable burden.

Thanks to methodical improvements, DNA adducts can be measured at ever lower levels. The danger to overestimate the contribution of a single genotoxic agent to the total background DNA damage increases whenever the limit of detection is lowered. The gap between the LOEL for adduct formation and the LOEL for biological endpoints widens correspondingly. DNA adduct measurement could dependent on the adduct and method use could be achived under best possible conditions down to levels on the order of 1 per 10^7 to 10^8. With the use of ^{14}C-labelled compounds measurements of levels even down to 1 per 10^{11} nt is achievable [150]. In comparison the human diploid genome contains approximately

9×10^9 nucleosides. Standard biological assays determining e.g. mutations are not nearly as sensitive. The suggestion is therefore to rather focus on further increasing quality and understanding of biological endpoints than further decreasing limits of detections in adduct analyses.

7.3.4 DNA adducts, genotoxicity assays and cancer risk

The induction of DNA adducts is an initial key event of numerous chemical carcinogens for tumor formation. DNA adducts are not adverse themselves, but require transformation into biological relevant lesions (chromosomal damage, mutations) to contribute significantly to the process of tumorigenesis. There are numerous mechanisms like DNA repair, that constantly reduce the amount of DNA adducts potentially processed into relevant lesions. In the view of this fact and of the complex events (genotoxic and non-genotoxic) that drive and prevent tumorigenesis, it appears not feasible that DNA adduct measurements could be used to predict tumor risk. DNA adducts overall can be used as a measure of exposure to target tissues. Specific DNA adducts involved in chromosomal damage and mutation induction can be further used as a measure of effective dose [14]. For DNA adducts to be used in a cancer risk assessment at low dose, DNA adducts have to be related to more general markers of genotoxicity.

In the present work an *in vitro* system was described that allowed to compare adduct levels with quantitative endpoints of genotoxicity such as DNA strand breaks, micronuclei and mutant frequency. For the assessment of the frequency of induced mutants, the gene used in the present work was that of the thymidine kinase. This particular locus is not known or predicted to be involved in tumorigenesis. However, the potential of adducts to induce mutations must in general be seen as a critical event significant for tumorigenesis.

Micronuclei determined e.g. in lymphocytes are not *per se* predictors of adverse health outcome at the level of an individual analyzed. However, a recent large-scale prospective study by Bonassi et al. has shown that individuals at the higher quantiles of micronuclei were at greater risk of developing cancer [38].

7.4 Background oxidative DNA damage in chemical carcinogenesis

The role of the NADPH oxidase on background levels of 8-oxodGuo and εdAdo *in vivo* was demonstrated using *p47phox*$^{-/-}$ mice and their *wt* counterparts. Since both adducts show promutagenic properties altered levels may play a significant role in carcinogenesis that involves inflammatory responses as e.g. shown for chemically-induced hepatocellular carcinoma [44]. Prior to the appearance of hepatocellular carcinoma liver cirrhosis is frequently observed, a process associated with high levels of cytotoxic damage in hepatocytes but also with inflammation and oxidative stress. Kupffer cells are the macrophages of the liver and are important in the regulation of inflammatory events as well as the elimination of pathogens. Diethylnitrosamine was also shown to activate Kupfer cells resulting in the release of (nonspecific) proinflammatory and cytotoxic mediators like cytokines (TNFα) or superoxide. The absence of a functional *p47phox*$^{-/-}$ abolishes superoxide generation in Kupffer cells [47] and *p47phox*$^{-/-}$ mice are largely protected from diethylnitrosamine induced hepatocarcinogenesis.

Diethylnitrosamine is a representative chemical of a family of carcinogenic *N*-nitroso compounds found at the workplace, in processed meats, as well as in tobacco smoke [151]. It may also be derived from metabolism of some therapeutic drugs. Ethanol is classified as a *known human carcinogen* [152]. In Southern Germany 50% of hepatocellular carcinoma patients have a history of ethanol abuse in the absence of hepatitis [45]. There is evidence that ethanol is neither directly genotoxic, mutagenic nor clastogenic *in vitro* [130], therefore ethanol was considered to contribute to tumor promotion rather than to initiation.

Chemical carcinogenesis is generally considered to be a multi-step process. Tumor initiation by induction of mutations is followed by tumor promoting effects mediated e.g. by enhanced cytotoxicity and cell death that results in regenerative cell proliferation that supports clonal expansion of initiated cells that may have acquired a growth advantage. The presented work suggests that tumor promoting effects of ethanol might also go along with increases in the oxidative DNA lesions 8-oxodGuo and εdAdo i.e. genotoxicity indirectly mediated by an inflammatory host response. This implies that at least in combination with a potent tumor initiator like diethylnitrosamine, ethanol exposure might also contribute indirectly to genotoxicity by affecting background levels of DNA adducts.

Morphological and histopathological analysis are under way at the Institute of Cancer Research (Vienna, Austria) and will help to characterize the hepatic response in more detail with respect to inflammation, necrosis and apoptosis. The ultimate effect of a chronic inflammatory host response involving superoxide generation, however, is complex and includes further host interactions like DNA repair, necrosis, regenerative hyperplasia (to name a few). It also depends on the regulation of local concentrations of superoxide, the microenvironment and on the genetic background of the individual (polymorphisms). The *p47phox*$^{-/-}$ mice model showed the possible role of superoxide generated by NADPH oxidase during an inflammatory response as a host factor in carcinogenesis. This model may be of use in intervention studies to evaluate the use of antioxidants or inhibitors of pro-oxidant enzymes for the prevention of cancer.

8 Summary

DNA adduct formation is a fundamental mechanism of chemically-induced mutagenesis and carcinogenesis. DNA adducts are the result of the primary interaction of a chemical carcinogen with DNA and require translation into subsequent biochemical or biological consequences (e.g. DNA strand breaks, chromosomal damage or mutations) in order to be biologically significant. Whether or not an adduct is converted into such a lesion depends on factors like the miscoding properties of the adduct but also on cellular responses like DNA repair, effects on the cell cycle or cell death. Some adducts are also formed as background from endogenous and unavoidable exogenous sources. Although DNA repair is constantly active, a steady-state level is present all the time and may be as high as one lesion per 10^5 normal nucleotides [18]. In view of this remarkable number, the biological significance of small exposure-related increments of DNA adducts is under discussion. Key questions pertain not only to the quantitative relationship between background adduct levels and exposure-related increments but also to the specific consequences of different adducts.

To provide accurate and reproducible quantification of DNA adducts at background and induced levels, appropriate analytical techniques are required. Therefore, in a first step of the presented work sensitive and specific liquid chromatography tandem mass spectrometry (LC-MS/MS) methodology for the quantification of the DNA adducts 7-methylguanine (7-mG), O^6-methyl-2´-

deoxyguanosine (O^6mdGuo), 8-oxo-7,8-dihydro-2´-deoxyguanosine (8-oxo-dGuo), and 1,N^6-etheno-2´-deoxyadenosine (εdAdo) was developed and validated for measurements in DNA isolated from tissue or cultured cells. These adducts were chosen because they are formed both endogenously and exogenously by DNA-methylation, direct oxidation of DNA, and reaction of DNA with lipid peroxidation products. The adducts O^6mdGuo, 8-oxo-dGuo and εdAdo could be analyzed after enzymatic hydrolysis as 2′-deoxyribonucleosides using on-line sample preparation by a column switching approach. O^6-mdGuo and its stable isotope-labeled analogue O^6-[$^2H_3$]mdGuo were synthesized, to be used as working standard and internal standard, respectively. Method validation was conducted in an artificial matrix consisting of the 4 normal 2′-deoxyribonucleosides (21% of each dGuo and dCyd, and 29% of each dAdo and dThd), similar to a hydrolysate of mammalian DNA. The validation parameters dynamic range, limit of detection, limit of quantification, inter-day precision and accuracy were evaluated. Since depurination of 7-mG from DNA is a continuous process, losses during the hydrolysis can take place. Therefore, a separate LC-MS/MS assay for 7-mG after thermal depurination from DNA was developed and validated. The methods were successfully applied to DNA isolated from rat liver (chapter 3), cultured L5178Y mouse lymphoma cells (chapter 5) and mouse liver (chapter 6).

DNA strand breakage as a possible consequence of DNA adducts can be assessed by the comet assay (single-cell gel electrophoresis). The alkaline version of this assay is capable detecting DNA single- and double strand breaks, alkali labile and abasic sites. Single cells are embedded into agarose and lysed, which is followed by electrophoresis. The DNA damage is estimated by the amount of DNA fragments that can migrate away from unbroken nuclear DNA during electrophoresis. After DNA staining this results in "comet-like" microscopic images. DNA fragmentation also occurs during cell death and may confound the comet assay analysis as a quantitative endpoint of genotoxicity. To determine whether effects in the comet assay could be explained by apoptosis and are therefore falsely interpreted as genotoxic DNA damage, three different chemicals with different modes of action were used. L5178Y mouse lymphoma cells were treated with (i) sodium arsenite ($NaAsO_2$) as a potent inducer of apoptosis exerting low mutagenicity (through largely unknown mechanisms), (ii) dexamethasone as non-genotoxic inducer of apoptosis [89,90] and (iii) the DNA methylating chemical methyl

methanesulfonate (MMS) as a genotoxic agent. Frequency histograms for single cell measures of % Tail DNA (percent of DNA migrated into the "comet" tail) were recorded in 30-minute intervals; the proportion of cells undergoing apoptosis was determined by annexin V staining in parallel. A time window was observed in which $NaAsO_2$ effectively triggered apoptosis and simultaneously gave comet assay results that resembled that of typical genotoxicity of MMS. The signal for $NaAsO_2$ changed time-dependently and at later times showed the typical bimodal distribution of cells without DNA migration and cells with high levels of DNA fragmentation that was also observed after dexamethasone treatment. It was suggested that a portion of the cells showing moderate DNA migration were early apoptotic cells and precursors of the cell showing high fragmentation levels at later times and did not indicate genotoxicity of $NaAsO_2$. Within the 4 h period of treatment with the methylating agent MMS the comet assay signal shifted to higher levels, but neither a bimodal distribution of damaged cells nor increased apoptosis was observed. In conclusion, comet assay analyses should be accompanied by an assay for early apoptosis in order to safeguard against misinterpretation.

The biological significance of DNA adducts depends on the biochemical and biological consequences of different adducts, the probability for the primary DNA damage to be expressed as heritable genetic changes and – in view of background DNA damage – on the quantitative relationship between background adduct levels and exposure-related increments. In other words, to what extent do increments in adducts result in increments in subsequent endpoints of genotoxicity and mutagenicity. Two methylating genotoxins with different modes of action were used to investigate background DNA damage and dose-related increments in L5178Y mouse lymphoma cells. Methyl methanesulfonate (MMS) reacts through a mechanism of S_N2-type mechanism whereas N-methyl-N-nitrosourea (MNU) MMS reacts via an mechanism of S_N1-type. Large batches of L5178Y mouse lymphoma cells were treated with MMS or MNU at equimolar concentrations. The formation of guanine methylation (7-mG and O^6-mdGuo) was determined by LC-MS/MS. In parallel, using the same population of treated cells genotoxicity assays were performed. The comet assay, the micronucleus test and the mouse lymphoma thymidine kinase mutation assay were used to determine DNA breakage, chromosomal damage and mutant frequency, respectively. The predominant adduct was 7-mG with both agents. MMS was slightly more potent than MNU at low dose,

much more potent at high dose. For methylation at O^6 of guanine, MNU was about five times more potent than MMS. MMS treatment induced strand breaks, alkali-labile sites and micronuclei with higher potency than MNU. This indicated that the biological consequences of S_N2-type alkylations are DNA breakage and chromosomal damage rather than gene mutation. The opposite holds for S_N1-type reactivity, as indicated by the higher potency of MNU for the induction of O^6-adducts and thymidine kinase gene mutations. This relation may be explained by the high probability of mispairing of O^6-mdGuo with thymidine during DNA replication. Furthermore, the result showed that the relative increase above background was much steeper for the methylations than for the endpoints to which these adducts contribute. In quantitative terms, the MMS or MNU concentration necessary to double the biological endpoint of DNA strand breakage was 10 to 15 times higher than the concentration that results in a doubling of the "chemical endpoint" DNA methylation. In conclusion, DNA-adduct formation as a "first-line" product of the interaction of a genotoxic agent with DNA may lead to a many-fold overestimation of the treatment-related biological relevance for more complex endpoints.

The final part of this work was devoted to analyses of background oxidative DNA adducts (8-oxodGuo and εdAdo) in NADPH oxidase knockout (*p47phox*$^{-/-}$) mice and their wild type (*wt*) counterparts. Lack of the p47phox gene abolishes superoxide anion ($O_2^{\bullet-}$) production in Kupffer cells. Since *p47phox*$^{-/-}$ mice are less sensitive to diethylnitrosamine induced hepatocarcinogenesis the question was addressed whether $O_2^{\bullet-}$ production affects background levels of promutagenic oxidative DNA lesions. Tumorigenesis was initiated in 6 week old mice followed by subsequent promotion by providing ethanol with the drinking water. Levels of 8-oxodGuo and εdAdo were measured in DNA isolated from liver samples using the LC-MS/MS method with on-line sample preparation by column switching. The 8-oxodGuo and εdAdo levels were significantly lower in *p47phox*$^{-/-}$ compared to *wt* mice. This demonstrates that $O_2^{\bullet-}$ production of Kupffer cells contributes to the background level of promutagenic oxidative DNA lesions in the liver and that endogenous or unavoidable exogenous sources of oxidative DNA damage other than $O_2^{\bullet-}$ from Kupffer cells exist. Treatment with diethylnitrosamine or ethanol alone did not result in an obvious increase in 8-oxodGuo and εdAdo lesions, neither in *p47phox*$^{-/-}$ nor in *wt* mice. Significant increases in 8-oxodGuo and εdAdo levels were observed after

the combination treatment (initiation with diethylnitrosamine and promotion with ethanol) in *wt* and less so in *p47phox*$^{-/-}$ mice.

DNA adducts mediated by $O_2^{\bullet-}$ from Kupffer cells was increased in hepatocytes during liver damage by diethylnitrosamine and chronic ethanol treatment. Chronic ethanol exposure might therefore not only contribute to tumor promotion but also indirectly to tumor initiation and progression. The data obtained with the *p47phox*$^{-/-}$ mice model suggest a possible role of superoxide generated during a inflammatory response as a host factor in carcinogenesis. This model may therefore also be of use in intervention studies to evaluate the effect of antioxidants or inhibitors of pro-oxidant enzymes for the prevention of cancer.

In conclusion, LC-MS/MS methodology for DNA adduct analyses was developed to determine DNA adducts at background levels and to evaluate treatment-related increases. In view of the fact that DNA damage occurs in living cells constantly, the question about the biological significance of DNA adducts must be discussed for treatment-related increments in relation to unavoidable background DNA damage and its variability. An experimental system was presented using L5178Y mouse lymphoma cells to compare increases in DNA methylations with increments in cellular consequences such as DNA breakage and mutations. To estimate the carcinogenic effect of a DNA-reactive agent, the evaluation of DNA adducts as a "first-line" interaction product of the carcinogen with its target may lead to an overestimation of the total effect. To improve cancer risk assessment at low-level exposures to carcinogens, sensitive biomarkers of biological effect are required rather than lowering the limits of detection of DNA-adduct analyses. Chemical carcinogenesis involves more than DNA damage and mutagenesis. It is a highly complex process, which is modulated by both genetic and epigenetic factors. Complex host responses (e.g. endocrine and immune responses) are also involved and may influence the outcome of an exposure to chemical carcinogens. As one important host factor in hepatocarcinogenesis, the role of Kupffer cells was investigated in liver of *p47phox*$^{-/-}$ compared to *wt* mice. It showed that $O_2^{\bullet-}$ generation by Kupffer cells constitutes a host factor in carcinogenesis induced by diethylnitrosamine and ethanol in that it modulates background levels of DNA damage. Further effort should go into the analysis of the variation of background DNA damage and the relationship between early biomarkers and clinical endpoints.

9 Zusammenfassung

Die Bildung von DNA-Addukten ist ein fundamentaler Mechanismus der chemischen Kanzerogenese. DNA-Addukte sind primäre Produkte der Interaktion eines chemischen Kanzerogens mit DNA. Um einen biologisch relevanten Effekt zu haben, müssen sie in biochemische und biologische Konsequenzen (z. B. DNA Strangbrüche, chromosomale Schäden) umgewandelt, oder letztlich als Mutationen fixiert werden. Ob und wie DNA-Addukte biologisch relevant werden, hängt von verschiedenen Faktoren ab, wie z. B. dem Fehlpaarungspotenzial aber auch von zellulären Reaktionen, wie der DNA-Reparatur oder von Effekten auf den Zellzyklus oder Zelltod. Manche DNA-Addukte werden auch als Hintergrund gebildet, durch endogene, aber auch durch nicht vermeidbare exogene Faktoren. Obwohl ständig DNA-Reparatur aktiv ist diesen Schaden zu reduzieren, stellt sich ein stabiler Gleichgewichtszustand ein, und erreicht unter Umständen Werte bis zu einem DNA-Addukt pro 10^5 normale Nukleotide. In Anbetracht dieses erheblichen Hintergrund-DNA-Schadens entstand eine Diskussion über die Relevanz von kleinen Inkrementen an chemisch-induzierten DNA-Addukten. Die Hauptfrage betrifft dabei die quantitative Beziehung zwischen der Höhe des Hintergrund-Addukt-Pegels und induzierten Inkrementen durch Exposition, aber auch die spezifischen Konsequenzen der unterschiedlichen DNA-Addukte selbst.

Um Hintergrund- und induzierte DNA-Addukte genau und reproduzierbar zu quantifizieren, benötigt man geeignete chemisch-analytische Techniken. Im ersten Schritt dieser Arbeit wurden deshalb empfindliche und spezifische Methoden, basierend auf Flüssigkeitschromatographie und Massenspektrometrie, für die Addukte 7-Methylguanin (7-mG), O^6-Methyl-2′-Desoxyguanosin (O^6mdGuo), 8-oxo-7,8-Dihydro-2′-Desoxyguanosin (8-oxo-dGuo), and 1,N^6-Etheno-2′-Desoxyadenosin (εdAdo), entwickelt und validiert. Diese Addukte wurden ausgewählt, weil sie sowohl endogen, als auch exogen durch DNA-Methylierung, direkte Oxidation von DNA und durch Reaktion von DNA mit Peroxidationsprodukten von Fettsäuren gebildet werden. Nach enzymatischer Hydrolyse der DNA zu den 2′-Desoxyribonukleosiden, konnten die DNA-Addukte O^6-mdGuo, 8-oxo-dGuo und εdAdo mit einer Säulenschaltung und einem On-line Probenvorbereitungssystem analysiert werden. Der Standard O^6-mdGuo und der interne Standard, das deuterierte Analogon O^6-[$^2H_3$]mdGuo, wurden synthetisiert. Die Validierung der Methode wurde in einer artifiziellen Matrix durchgeführt, um Matrix-Effekte zu kompensieren. Diese bestand aus einem Gemisch der vier 2′-Desoxyribonukleosiden (dGuo und dCyd jeweils 21%, dAdo und dThd jeweils 29%), vergleichbar einem Hydrolysat von Säuger-DNA. Die Methode wurde bezüglich der Parameter, dynamischer Bereich, Nachweisgrenze, Quantifizierungsgrenze, Präzision und Genauigkeit validiert. Nachdem 7-mG als Folge von Depurinierung spontan freigesetzt wird, können Verluste an 7-mG, die während der Probenaufbereitung zu den 2′-Desoxyribonukleosiden entstehen nicht reproduzierbar quantifiziert werden. Deshalb wurde für die Analyse von 7-mG eine separate Methode nach thermischer Depurinierung von DNA entwickelt und validiert. Die LC-MS/MS Methoden wurden erfolgreich angewandt an DNA aus Rattenleber (Kapitel 3), aus kultivierten L5178Y Maus Lymphomzellen (Kapitel 5) und aus Mausleber (Kapitel 6).

DNA-Strangbrüche als eine mögliche Folge von DNA-Addukten können mit dem Kometen-Test (auch Einzelzell-Gel-Elektrophorese) bestimmt werden. Die alkalische Version dieses Tests detektiert Einzel- und Doppelstrangbrüche, alkali-empfindliche und basenfreie Stellen. Vor der Elektrophorese werden einzelne Zellen in Agarose eingebettet und lysiert. Kleinere DNA-Fragmente können schneller wandern als nicht oder wenig geschädigte DNA und bilden entsprechend Ihrer Ladung und Größe einen „Kometenschweif“ der durch Anfärbung sichtbar gemacht wird. Die

Abschätzung des DNA-Schadens basiert auf der im Schweif enthaltenen DNA-Menge.

DNA-Strangbrüche treten auch aufgrund von zytotoxischen Prozessen auf. Das kann die Interpretation von Kometen-Analysen als quantitativen Endpunkt für Gentoxizität erschweren. Ein Ziel war es deshalb zu untersuchen, ob Effekte im Kometen-Test auf der Basis von Apoptose erklärt werden können, und somit als gentoxischer Schaden fehlinterpretiert werden. Es wurden drei verschiedene Testsubstanzen mit unterschiedlicher Wirkungsweisen in L5178Y Maus Lymphomzellen untersucht. (i) Natriumarsenit ($NaAsO_2$) als ein Agens, welches Apoptose induziert und schwach mutagen ist, (ii) Dexamethason, als ein nicht-gentoxisches, apoptose-induzierendem Agens und (iii) Methylmethansulfonat (MMS), ein DNA methylierendes Agens. In 30-Minuten Intervallen wurde über vier Stunden die Häufigkeitsverteilung des Anteils DNA, der in den Schweif gewandert ist (% Tail DNA) für Einzelzellen bestimmt. Parallel dazu wurde mit Annexin-V-Färbung der Anteil Zellen bestimmt, in denen Apoptose induziert wurde. Es zeigte sich ein Zeitfenster, in welchem $NaAsO_2$ Apoptose induzierte aber gleichzeitig Effekte im Kometen Test zeigte, welche dem Signal eines typisch gentoxischen Agens (MMS) ähnlich waren. Das Signal für $NaAsO_2$ änderte sich im Kometen-Test zeitabhängig von wenig zu moderater DNA-Migration und wechselte an späteren Zeitpunkten zu einer bimodalen Verteilung von Zellen mit fehlender DNA-Migration und Zellen mit hohem Grad an DNA-Fragmentierung. Diese charakteristische Verteilung von Zellen wurde auch nach Behandlung mit Dexamethason beobachtet. Daraus wurde geschlossen, dass ein Teil der Zellen mit moderaten Grad an DNA-Migration tatsächlich apoptotische Zellen in frühen Stadien waren und somit möglicherweise Vorläufer jener Zellen, die später die starke DNA Fragmentierung zeigten. Diese Zellen wären somit keine Indikatoren für Gentoxizität durch $NaAsO_2$. Das methylierende, gentoxische Agens MMS dagegen zeigte mit der Zeit eine Verschiebung des Kometen-Test-Signals zu höheren Werten, aber weder eine bimodale Verteilung der Zellen noch erhöhte Induktion von Apoptose. Die Daten belegen, dass Analysen mit dem Kometen-Test durch einen Test auf frühe Stadien der Induktion von Apoptose ergänzt werden sollten, um Testergebnisse von zytotoxischen Prozessen abzugrenzen.

Die Beurteilung der biologischen Bedeutung von DNA-Addukten ist einerseits von den biochemischen und biologischen Eigenschaften der verschiedenen Addukte

abhängig, andererseits von den Anforderungen die nötig sind um den primären Schaden "DNA-Addukt" in einen genetischen, vererbbaren Schaden zu fixieren. In Anbetracht des Hintergrund-DNA-Schadens ist es wichtig, inkrementelle DNA-Addukte in Relation zum Hintergrund-DNA-Schaden zu erörtern. Die Frage ist, inwieweit Inkremente über Hintergrund in DNA-Addukten sich in Inkrementen in späteren Endpunkten von Gentoxizität und Mutagenität ausprägen. Dazu wurden zwei methylierende, gentoxische Agenzien mit unterschiedlicher Reaktivität gegenüber DNA herangezogen, und dosisabhängige Inkremente wurden in Relation zum Hintergrund-DNA-Schaden in L5178Y Maus Lymphomzellen untersucht. Reaktionen von Methylmethansulfonat sind vom S_N2-Typ während Methylnitroso-harnstoff dem S_N1-Typ entspricht. Große Ansätze von L5178Y Maus Lymphomzellen wurden mit äquimolaren Konzentrationen von MMS und MNU behandelt. Die Bildung der beiden Methylguanine 7-mG und O^6-mdGuo wurde mittels LC-MS/MS verfolgt. Parallel dazu wurden in derselben behandelten Zellpopulation drei quantitative gentoxikologische Tests zur Bestimmung von DNA-Strangbrüchen (Kometen-Test), chromosomalen Schäden (Mikrokern-Test) und von Genmutationen (Maus-Lymphom-Thymidin-Kinase-Test) durchgeführt. Das durch MMS und MNU überwiegend gebildete DNA-Addukt war 7-mG. Bei niedriger Dosis war MMS geringfügig potenter als MNU, jedoch deutlich potenter bei hoher Dosis. Für die Methylierung in Position O^6 von Guanin war MNU etwa fünfmal potenter als MMS. Die Behandlung mit MMS induzierte DNA-Strangbrüche und Mikrokerne mit größerer Potenz als MNU. Für die Induktion von Genmutationen war das Verhältnis umgekehrt, MNU war bei gleicher Konzentration potenter als MMS. Dies zeigte, dass DNA-alkylierende Substanzen vom S_N2-Typ eher DNA-Brüche und chromosomale Schäden induzieren, weniger dagegen Genmutationen. Bei S_N1-Reaktivität ist das Verhältnis umgekehrt. Die höhere Potenz von MNU, O^6-mdGuo und Genmutationen zu generieren, zeigt den Zusammenhang der hohen Potenz für Fehlpaarung von O^6-mdGuo (Thymidin statt Cytosin) während der DNA-Replikation und der Fixierung als Mutation. Außerdem zeigten die Ergebnisse, dass der Anstieg in DNA Addukten relativ zum Hintergrund viel steiler waren, als die Anstiege in den gentoxikologischen Endpunkten, zu denen die Addukte beitragen. Die Konzentration an MMS oder MNU, die nötig war, den Hintergrund an DNA-Strangbrüchen zu verdoppeln, war etwa 10 bis 15 mal größer, als die Verdoppelungsdosis des chemischen Endpunkts "DNA-Methylierung". Daraus folgt, dass DNA-Addukte als primäres DNA-Interaktions-

Produkt eines gentoxischen Agens keine vollwertigen Biomarker für spätere, biologische Endpunkte sein können.

Der abschließende Teil der Arbeit widmete sich der Analyse von oxidativen Hintergrund-DNA-Addukten in NADPH-Oxidase Knockout (p47phox$^{-/-}$) und Wildtyp-Mäusen. Die Knockout Mäuse sind bezüglich der Induktion von hepatozellulären Karzinomen durch Diethylnitrosamin weniger empfindlich. Die Frage war, ob die bei fehlendem p47phox Gen reduzierte Produktion des Superoxidradikalanions ($O_2^{\bullet-}$) durch Kupffer Zellen auch zu einer Reduzierung der oxidativen, promutagenen Hintergrund-DNA-Addukten führt.

Die Kanzerogenese wurde in 6 Wochen alten p47phox$^{-/-}$ und Wildtyp-Mäusen initiiert, zur Tumorpromotion wurde Ethanol im Trinkwasser verabreicht. Die Level von 8-oxo-7,8-Dihydro-2′-Desoxyguanosin (8-oxo-dGuo), und 1,N^6-Etheno-2′-Desoxyadenosin (εdAdo) wurden mittels der in Kapitel 3 beschriebenen LC-MS/MS Methode in der Leber gemessen. In p47phox$^{-/-}$ Mäusen waren die Werte für 8-oxodGuo und εdAdo signifikant niedriger als in Wildtyp-Mäusen. Dennoch war ein Hintergrund der beiden DNA-Addukte auch in p47phox$^{-/-}$ Mäusen klar messbar. Das bedeutet, dass $O_2^{\bullet-}$ aus Kupffer Zellen zwar zum Hintergrund der oxidativen DNA-Addukte beiträgt, dass aber außer $O_2^{\bullet-}$ noch andere, endogene oder unvermeidlich exogene Quellen für oxidative DNA-Schäden existieren.

Die Behandlungen mit Diethylnitrosamin oder Ethanol alleine resultierten in keinen klaren Änderungen der 8-oxodGuo and εdAdo Level, weder in p47phox$^{-/-}$, noch in Wildtyp-Mäusen. Signifikante Anstiege der 8-oxodGuo und εdAdo Level wurden dagegen nach der Kombinationsbehandlung mit Diethylnitrosamin und Ethanol beobachtet. Besonders ausgeprägt war dieser Synergismus in Wildtyp-Mäusen, weniger in den Knockout-Tieren. Dies könnte bedeuten, dass die durch $O_2^{\bullet-}$ vermittelten DNA-Addukte gentoxische Effekte in Hepatozyten im Zuge einer Leberschädigung verstärken. Durch die Erhöhung der Level von promutagenen DNA-Addukten könnte Ethanol in diesem Fall nicht nur zur Tumorpromotion, sondern auch zur Tumorprogression beitragen.

Das verwendete p47phox$^{-/-}$ Mausmodell zeigt eine mögliche Rolle des Superoxidradikalanions als Wirtsfaktor in der chemischen Kanzerogenese, vermittelt durch eine entzündliche Reaktion. In Interventionsstudien könnte dieses Model herangezogen werden, zum Beispiel zur Abschätzung der Wirkung von Antioxidantien oder Inhibitoren von pro-oxidativen Enzymen zur Krebsprävention.

In der vorliegenden Arbeit wurden sensitive und selektive LC-MS/MS Methoden entwickelt um DNA-Addukte, die sowohl als Hintergrund aber auch chemisch-induziert entstehen können, zu messen. Die Frage der biologischen Bedeutung von chemisch-induzierten DNA-Addukten wurde als Inkrement zum unvermeidlichen Hintergrund erörtert und bezüglich dessen Variabilität diskutiert. Ein experimentelles System basierend auf L5178Y Maus Lymphomzellen wurde dargestellt. Dies ermöglichte, Anstiege von induzierten DNA-Methylierungen mit Inkrementen in zellulären Konsequenzen wie z. B. DNA-Strangbrüche oder Mutationen zu vergleichen. Es wurde festgestellt, dass der kanzerogene Effekts eines DNA-reaktiven Agens überschätzt wird, wenn dies ausschließlich auf DNA Addukten basiert. Für eine verbesserte Voraussage von biologischen Effekten nach Exposition gegenüber DNA-reaktiven Kanzerogenen ist insbesondere im Niedrigdosis-Bereich ein besseres Verständnis der Biomarker der biologischen Effekte erforderlich. Dies wäre sinnvoller als eine weitere Reduktion der Nachweisgrenzen für DNA Addukte.

Chemische Kanzerogenese umfasst mehr als nur DNA Schädigung und Mutagenese. Es ist ein vielschichtiger Prozess, der von genetischen und epigenetischen Faktoren beeinflusst wird. Im besonderen in *in-vivo*-Situationen können komplexe wirtsvermittelte Reaktionen (z. B. hormonelle Einflüsse und Immunreaktionen) die Konsequenzen einer Belastung durch ein Kanzerogen beeinflussen. Am Beispiel von p47phox Knockout-Mäusen wurde die Rolle der Superoxidradikalanion-Bildung durch Kupffer Zellen als ein möglicherweise wichtiger Wirtsfaktor in der Entstehung von hepatozellulären Karzinomen beispielhaft aufgezeigt.

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(Andreas Brink)