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The Formation and Biological Significance of *N*7-Guanine Adducts

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Abstract

DNA alkylation or adduct formation occurs at nucleophilic sites in DNA, mainly the *N*7-position of guanine. Ever since identification of the first *N*7-guanine adduct, several hundred studies on DNA adducts have been reported. Major issues addressed include the relationships between *N*7-guanine adducts and exposure, mutagenesis, and other biological endpoints. It became quickly apparent that *N*7-guanine adducts are frequently formed, but may have minimal biological relevance, since they are chemically unstable and do not participate in Watson Crick base pairing. However, *N*7-guanine adducts have been shown to be excellent biomarkers for internal exposure to direct acting and metabolically activated carcinogens. Questions arise, however, regarding the biological significance for *N*7-guanine adducts that are readily formed, do not persist, and are not likely to be mutagenic. Thus, we set out to review the current literature to evaluate their formation and the mechanistic evidence for the involvement of *N*7-guanine adducts in mutagenesis or other biological processes. It was concluded that there is insufficient evidence that *N*7-guanine adducts can be used beyond confirmation of exposure to the target tissue and demonstration of the molecular dose. There is little to no evidence that *N*7-guanine adducts or their depurination product, apurinic sites, are the cause of mutations in cells and tissues, since increases in AP sites have not been shown unless toxicity is extant. However, more research is needed to define the extent of chemical depurination versus removal by DNA repair proteins. Interestingly, *N*7-guanine adducts are clearly present as endogenous background adducts and the endogenous background amounts appear to increase with age. Furthermore, the *N*7-guanine adducts have been shown to convert to ring opened lesions (FAPy), which are much more persistent and have higher mutagenic potency. Studies in humans are limited

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Supplemental Information: The supplemental tables are provided in Microsoft Excel format to allow sorting and incorporation into a database for searching if needed. These tables were the basis for our review. Given the numbers of publications, we are certain that this compilation will not be complete and apologize for any references that were not included. All values are given as adducts /10^7 normal nucleotides (nnt). In case other units were reported, values were converted as followed;

I. pmol adduct/ μmol Gua = 1.95 adducts/10^7 nnt

II. fmol/ μg DNA = 3.25 adducts/ 10^7 nnt

If data were reported graphically, values were estimated from figures. These values need to be used with some caution, as they do not represent exact values.

in sample size and differences between controls and study groups are small. Future investigations should involve human studies with larger numbers of individuals and analysis should include the corresponding ring opened FAPy derivatives.

Keywords

*N*7-guanine adducts; mutagenesis; biomarkers; exposure assessment; Dose-response

1 Introduction

DNA alkylation or adduct formation occurs at nucleophilic sites in DNA. Of these nucleophilic sites, the *N*7-position of guanine is the most reactive [1]. A PubMed search on "N7-guanine adducts" resulted in over 300 publications with 9 out of 10 focusing on basic characterization of chemical or biochemical properties of *N*7-guanine adducts alone or in DNA. In addition, *N*7-guanine adducts are classified as non promutagenic since they are chemically unstable and the *N*7-position does not participate in Watson Crick base pairing [2]. Ever since identification of the first *N*7-guanine adduct [1], several hundred studies on DNA adducts have been reported and reviewed from different perspectives [3-16]. Consequently, many studies sought to establish the relationship between DNA adduct formation and other biological endpoints (mutations, DNA strand breaks, *etc*.). Technical limitations, however, did not permit integration into large molecular epidemiological studies during this era of lesion characterization. Despite superior sensitivity of the ^{32}P-postlabeling assay, insufficient chemical specificity made it impossible to identify the chemical source of damage, and chemical depurination of *N*7-guanine adducts during sample preparation was a major concern. Almost all studies started with *in vitro* proof of concept experiments demonstrating covalent binding of the compounds of interest or their metabolites to DNA. Surprisingly, the identification and development of sensitive analytical methods remain a primary focus of many DNA adduct studies, even 50+ years later.

*N*7-guanine adducts appear to be good biomarkers of internal exposure because of their higher abundance compared to other DNA alkylations. Questions arise, however, regarding the biological significance for *N*7-guanine adducts that are readily formed, do not persist, and are not likely to be mutagenic. Thus, we set out to review the current literature to evaluate their formation and the mechanistic evidence for the involvement of *N*7-guanine adduts in mutagenesis or other biological processes.

1.1 Formation of DNA adducts

Miller and Miller pioneered the field of chemical carcinogens and were the first to demonstrate covalent binding of chemical carcinogens to macromolecules *in vivo* [17,18]. The first evidence for binding of chemical carcinogens or their metabolites to nucleic acid was reported by Wheeler and Skipper [19]. It quickly became apparent that carcinogens comprise a diverse group of chemicals. Some of them were from endogenous sources or natural products, while others arise from synthetic products of modern human life. These chemicals are able to react with nucleophilic sites (electron rich, S, N, and O), in DNA and proteins. Subsequent *in vitro* and *in vivo* studies quickly demonstrated that under physiological conditions (pH 7.4, 37° C), alkylation of DNA primarily occurred at the *N*7-position of guanine (Table 1) [20]. The distribution of methylation and ethylation adducts in DNA was studied in *in vitro* reactions, in bacterial or mammalian cell cultures, and in several tissues of mice and rats (reviewed by Beranek [8]). Overall, it confirmed the notion that the relative distribution of alkylation in DNA is similar *in vivo* and *in vitro* [21]. However, as technology advanced and allowed examination of lower exposures distinct differences in adduct distribution were established (see Section 2).

Binding was shown to mainly occur *via* monomolecular (S_N1, *e.g.*, nitrogen mustards) or bimolecular nucleophilic (S_N2, *e.g.*, sulfonyl esters) substitutions [22-24]. S_N2 reactions are heavily dependent on steric accessibility while S_N1 reactions generally follow first-order kinetics. In DNA, the ring nitrogens and the exocyclic oxygens are the preferred sites for alkylation. Although the *N*7-position is the major site of alkylation, the electrophilic species formed by the *N*-nitroso compounds for example, following S_N1 kinetics, will have a greater preference for reaction at the exocyclic oxygens than will the alkanesulfonates, which are limited to S_N2 reactions. The larger the alkyl group is, the stronger will be its preference for reaction at the O^6-position of guanine. Hence, *N*-ethyl-*N*-nitrosourea (ENU) binds more efficiently to the O^6-position than does *N*-methyl-*N*-nitrosourea (MNU) (Table 1)[8,25,26]. The important difference in alkylation agents undergoing S_N1 or S_N2 reactions is that agents capable of S_N1 reactions react more frequently at the O^6-position of guanine, thus producing more mutagenic O^6-guanine adducts, compared to agents that solely react *via* the S_N2 mechanism.

These early binding experiments in DNA, cell culture and animal studies also showed that some carcinogens required metabolic activation to gain their ability to form DNA adducts and to exhibit their mutagenic and carcinogenic effects. Consequently, compounds were classified as “direct-acting” or “metabolically activated” carcinogens. The latter type is also termed a pro-carcinogen. In addition to mono adducts, compounds with multiple reactive groups were shown to have the ability to form protein-protein, DNA-DNA or protein-DNA cross-links [20]. The decades following have produced a better understanding of the relationship between carcinogen exposures, DNA adduct formation, mutagenesis, and carcinogenesis [4-7,10,12, 27]. Various technologies have been applied to animal and human exposure studies for routine analysis of *N*7-guanine adducts and other DNA adducts. These studies have increased our understanding of formation and persistence of DNA adducts, and their relationship to carcinogenesis. It has become clear that cancer is a complex, multi-step process that varies with types of exposure, site of tumor induction, and species. Understanding the implications of *N*7-guanine adducts has significantly contributed to identification of the mode of action in chemically-induced mutagenesis and carcinogenesis. These findings have subsequently led to a better understanding of the role of DNA adducts in mutagenesis and mechanism-based risk assessment [27].

1.2 Stability of N7-guanine adducts

Compared to many other DNA adducts, *N*7-guanine adducts are chemically unstable, with half lives in double-stranded DNA (dsDNA) ranging from as little as 2 h to 150 h. The instability of *N*7-guanine adducts is created by the formal placement of an additional positive charge on the guanine ring system. In general, larger alkyl groups promote depurination in dsDNA. This has been demonstrated under physiological conditions (pH 7.4, 37°C), where the half-lives for *N*7-methyl-guanine (*N*7-Me-Gua), *N*7-(2-hydroxy-3-butene)-guanine (*N*7-HB-Gua) and *N*7-(trihydroxy-benzo[a]pyreneyl) guanine are 150 h, 50 h, and 3 h, respectively [28-31]. In addition, *N*7-guanine adducts accumulate in DNA with continuous exposure or treatment and usually reach a plateau (steady state) after ~7-10 days [15,32-34]. Steady state is reached when the rate of *N*7-guanine adducts formed is equal to the rate of adducts lost. In contrast, adducts that are more persistent, such as O^4-ethyl-thymidine (O^4-Et-Thy), accumulate over a period of 4 weeks [35], and O^6-methyl-guanine (O^6-Me-Gua) in the brain continue to accumulate over 6 weeks of dosing [36]. The formal placement of an additional positive charge on the guanine ring system also promotes further reactions that have been reviewed by Gates *et al.* [37]. Relative to guanine, *N*7-Me-Gua depurinates 10^6 times more rapidely at pH7, 37 °C [37]. Reactions characteristic for *N*7-guanine adducts are: (i) loss of C-8 proton, (ii) depurination, (iii) ring opening to yield 5-*N*-alkyl-2,6,-diamino-4-hydroxyformamidopyrimidine (alkyl-FAPy), (iv) hydrolysis of the *N*7-alkyl bond, and (v) rearrangement to C8 adducts. For details

of the chemical reactivity of N7-guanine adducts, the reader is referred to the thorough review by Gates [37].

1.3 Methods for detection of *N*7-guanine adducts

During the past 50 years many technologies have been used for analysis of *N*7-guanine adducts. These technologies have been applied to rodent and human exposure studies for routine analysis of *N*7-guanine adducts and other DNA adducts. In the earliest studies, radiolabeled carcinogens were administered to rodents and binding to protein, RNA, and DNA was assessed by scintillation counting of the corresponding cellular fractions [38]. After DNA isolation and hydrolytic treatments, individual DNA adducts could be purified and quantified by basic column chromatography. This approach allowed analysis of one sample per day, with a detection limit of 1 adduct per 10^6 normal nucleotides (nnt) using ≥5 mg DNA [29,39]. Longer exposure regimes were laborious and expensive, due to the requirement of radiolabeled carcinogen for these studies. Consequently, most studies employed single exposures [40,41].

By the 1980's, HPLC with fluorescence detection, radioimmunoassay, or enzyme-linked immuno sorbent assay were commonly used for the analysis of DNA adducts. These approaches significantly increased throughput, reduced cost *via* elimination of custom radioisotope synthesis, and allowed application to study designs that included multiple exposure protocols. The extended exposure protocols provided information on the steady-state concentrations of DNA adducts and demonstrated that what had previously appeared to be minor adducts following single exposures could actually become major adducts if they were poorly repaired and accumulated with extended exposure [42]. These methods, however, had limited sensitivity compared to present day technology and some of the immunoassays were prone to false positive results due to cross-reactivity.

A major break though in methodology occurred in 1982, when Randerath and colleagues developed ^{32}P-postlabeling methods for DNA adducts [43]. The limit of detection for the early ^{32}P-postlabeling assays was 1 adduct per 10^8 nnt using as little as 1-2 μg DNA [43,44]. Subsequently, combinations of ^{32}P-postlabeling with HPLC or immunoaffinity permitted larger amounts of DNA to be analyzed and improved the sensitivity by one or more orders of magnitude. The major problems associated with this methodology include the lack of chemical-specific identity and poor reproducibility [45,46]. The ^{32}P-postlabeling method was most suitable for more stable DNA adducts, such as etheno adducts [47-49] and N^2-guanine adducts derived from polycyclic aromatic hydrocarbons (PAH) [50], and less so for *N*7-guanine adducts, due to their instability.

More recently, advances in mass spectrometry have lowered the limit of detection for this chemical-specific and quantitative technology, making it the method of choice in contemporary investigations. Earlier studies applied gas chromatography - negative ion chemical ionization - mass spectrometry (GC-MS), after hydrolysis and derivatization, to the analysis of DNA adducts [51-56]. Presently, however, the vast majority of quantitative analysis of DNA adducts is performed with liquid chromatography tandem mass spectrometry (LC-MS/MS). The application of mass spectrometry for DNA adducts has been recently reviewed by Singh and Farmer [57] and others [15,16,58-63]. Major advances in instrumentation for both mass spectrometry and chromatography have increased the detection limits for DNA adducts up to 100-fold, making it possible to routinely measure 1 adduct per 10^8 nnt. A major advantage of GC- and LC-MS/MS methods is the utilization of chemically identical stable isotope labeled internal standards for accurate quantitation.

The greatest sensitivity for measuring DNA adducts, however, is achieved with accelerator mass spectrometry (AMS), which can quantitate down to 3 adduct per 10^{11} nucleosides using 1 μg DNA [64]. While this method is extremely sensitive, it is limited to the following

radioisotopes (^{3}H, ^{14}C, ^{26}Al, ^{41}Ca, ^{10}Be, ^{36}Cl, ^{59}Ni, ^{63}Ni, and ^{129}I), of which ^{14}C and ^{3}H are the most commonly used in biomedical research. Therefore, specific chemical syntheses are required to either obtain radioisotope-labeled test compounds or for chemical labeling of compounds of interest (postlabeling, derivatization). Unfortunately, access to AMS is limited worldwide (only 5 instruments as of 2007), mainly due to the expense of the mostly custom-made instruments [65].

2 Formation of *N*7-guanine adducts in animal models

Several investigators have successfully utilized *N*7-guanine adducts as biomarkers to answer important toxicology questions in rodent models. These studies used multi-dose exposure protocols to generate comprehensive dose-response curves. Data from studies in mice and rats are presented in supplemental materials (Table S1 and Table S2). Adduct formation was compared to other biological endpoints such as unscheduled DNA synthesis, mutation frequency, micronucleus, apurinic sites (AP sites), gene expression, and others [7, 10, 12, 66]. In the following sections we will describe studies demonstrating factors influencing the formation and persistence of *N*7-guanine adducts. First, metabolic activation and subsequent formation of *N*7-guanine adducts can be species, strain, tissue, and cell dependant [32, 67-69]. Second, chemical stability and DNA repair influence the ratios of *N*7-guanine adducts to other DNA adducts [36, 40]. Lastly, there is some evidence for accumulation and increased tolerance to, and higher formation rates of *N*7-guanine adducts in DNA at later ages compared to young ages [70, 71].

2.1 Nitrosourea compounds

N-methyl-*N*-nitrosourea (MNU) primarily induced tumors in the nervous system in rats and lymphoid tumors in mice [72-74]. Subsequent analysis of *N*7-Me-Gua in several tissues of MNU-treated A/J and C3H3B/FeJ mice showed the highest formation of *N*7-Me-Gua and *O*^{6}-methyl-guanine (*O*^{6}-Me-Gua) in liver, kidney, lung, and brain, suggesting that adduct formation might be involved in carcinogenesis (Figure 1) [75]. Comparison of the ratio of *N*7-guanine adducts to *O*^{6}-guanine adducts in brain and liver, assuming no active repair for *N*7-Me-Gua, suggested somewhat slower repair of *O*^{6}-Me-Gua in the more susceptible C3H3B/FeJ strain than in A/J mice [75]. Persistence of *N*7-Me-Gua was similar in liver (non-target tissue) and brain (target tissue), suggesting that *N*7-Me-Gua adducts are most likely not causally linked to mutagenesis and carcinogenesis. *O*^{6}-Me-Gua adducts, however, were removed much faster in liver than in brain, demonstrating active removal in the liver and suggesting involvement of *O*^{6}-Me-Gua in mutagenesis and carcinogenesis [76]. In contrast, the persistence and amounts of *O*^{6}-Me-Gua were similar in brain of both rats and mice, although these species differ markedly in their susceptibility to brain tumorigenesis, with the rat being a much more susceptible species [73,77]. It was concluded that organotropic carcinogenic effects of methylating carcinogens do not solely depend on DNA adduct formation and persistence, since formation and persistence correlated only in certain cases with tumor formation [75,78,79].

From the earliest studies on *N*7-guanine adducts, it quickly became apparent that certain adducts exist in control DNA and the notion of endogenous DNA damage was established. Amounts of *N*7-Me-Gua were 2-fold higher in liver DNA of rats that were 24 months old compared to 6 month-old animals, suggesting accumulation with age [71]. Similarly, endogenous amounts of *N*7-Me-Gua were twice as high in 29 month-old C57BL/6NNia mice, compared to 11 month-old mice [70]. More importantly, not only was the endogenous amount higher in older mice (29 month), they also formed twice as many *N*7-Me-Gua adducts as younger mice after 25 or 50 mg/kg MNU-treatment (11 months) [70]. These two reports demonstrate that endogenous background amounts and adduct formation may be significantly different at different ages. Unfortunately, to our knowledge age dependence of *N*7-guanine adduct formation has not been systematically investigated. Future studies should include

establishing endogenous background amounts of *N*7-guanine adducts and their formation in target tissues at different ages in mice, rats, and humans.

N-Ethyl-*N*-nitrosourea (ENU), similar to MNU, is a potent carcinogen inducing mainly tumors of nervous system and forming *N*7-ethyl-guanine (*N*7-Et-Gua) and O^6-ethyl-guanine (O^6-Et-Gua) adducts. The persistence of *N*7-Et-Gua adducts is dependent upon their chemical stability and possible elimination by active DNA repair enzymes. It has been shown that DNA repair capacity is tissue-specific and influences accumulation of promutagenic O^6-Et-Gua, O^4-Et-Thy and O^2-ethyl-thymidine (O^2-Et-Thy) adducts, but not *N*7-Et-Gua adducts [40]. Tissue-specific DNA repair can greatly affect dose-response of DNA adducts as exemplified with O^6-Me-Gua and O^6-Et-Gua in brain and liver of MNU- or ENU-treated mice or rats [36,40, 41]. O^6-Me-Gua and O^6-Et-Gua adducts are actively repaired in liver but not in the brain, the target tissue of MNU and ENU tumorigenesis in rats [80]. After a single exposure, O^6-guanine adducts are readily removed in the repair-proficient hepatic tissue but remain relatively persistent in the repair-deficient brain tissue. In contrast, the chemically less stable *N*7-Et-Gua and *N*3-Et-adenine adducts have similar elimination rates in both tissues, suggesting that these adducts are lost due to spontaneous depurination [40,41]. Consequently, the ratio of *N*7-Et-Gua to O^6-Et-Gua decreases in target tissues with time and duration of exposure, and the presence of *N*7-guanine adducts does not predict a fixed amount of O^6-guanine adducts. Most importantly, the presence of *N*7-guanine adducts cannot serve as quantitative indicator for the existence of other mutagenic or genotoxic lesions, without reliable knowledge of the half-lives in tissue and time since exposure. Although ratios of formation of related adducts can be expected to following *in vitro* chemistry, tissue-specific differences in repair and stability of the individual adducts will alter adduct-distribution and this dynamic must be considered in subsequent interpretations.

2.2 Nitrosamines

Nitrosamines are a class of chemical compounds that were first described in the chemical literature over 100 years ago, but not until 1956, did they receive much attention. During a routine screening of chemicals that were being proposed for use as solvents in the dry cleaning industry, John Barnes and Peter Magee, reported that dimethylnitrosamine (DMN) produced liver tumors in rats [81]. Magee and Barnes' landmark discovery caused scientists around the world to investigate the carcinogenic properties of other nitrosamines and *N*-nitroso compounds. Approximately 300 of these compounds have been tested, and 90% of them have been found to be carcinogenic in a wide variety of experimental animals, with many of them exhibiting organ specificity in their carcinogenicity [74]. For instance, DMN causes liver, kidney, and lung cancer in experimental animals, and some of the tobacco-specific nitrosamines are very potent pulmonary carcinogens [82-85]. *N*-nitrosamines require metabolic activation to exhibit their mutagenic and carcinogenic effects. Metabolic activation of nitrosamines is catalyzed by various forms of P450 enzymes in reactions that in general form a highly reactive diazonium ions and aldehydes [86]. Both the diazonium ions and the aldehydes form DNA adducts, including *N*7-guanine adducts (Figure 2).

***N*,*N*-Dimethylnitrosamine (DMN)** is potent rodent carcinogen inducing liver, kidney, and lung tumors, with mice being more susceptible than rats [81,87]. To investigate potential mechanisms responsible for species differences, the formation of *N*7-Me-Gua and O^6-Me-Gua were determined in mice and rats treated with DMN [88]. The experimental results of this study indicated distinct differences in rat and mouse hepatocytes for repair of the promutagenic DNA lesion, O^6-Me-Gua. The ratio of *N*7-Me-Gua to O^6-Me-Gua indicated similar repair of O^6-Me-Gua at low DMN exposures in rats and mice. However, the ratios of *N*7-Me-Gua to O^6-Me-Gua changed with increasing DMN dose, suggesting differential ability of these 2 species to repair increasing amounts of O^6-Me-Gua. This difference was due to readily inducible O^6-

methylguanine DNA-methyltransferase (O^6MT), found in the rat liver, but not the mouse. Pegg and Hui [89] first observed that the dose-response curves for O^6-Me-Gua induced by DMN were different from that of *N*7-Me-Gua. Studying DNA adducts derived from DMN, it was noticed that the amounts of O^6-Me-Gua were much lower relative to *N*7-Me-Gua at low exposures than at high exposures. The ratio of *N*7-Me-Gua to O^6-Me-Gua at high doses was approximately 10, while at lower exposure the amounts of *N*7-Me-Gua were about 100-fold higher than that of O^6-Me-Gua [89]. The change in ratio of *N*7-Me-Gua to O^6-Me-Gua was later shown to be a result of depletion of O^6MT at high doses. O^6MT is a highly efficient and specific DNA repair protein that selectively repairs O^6-Me-Gua. O^6MT removes the alkyl groups from the O^6-guanine position by transferring them to the protein itself, effectively restoring the guanine base in DNA, and thereby inactivating the protein [90]. Thus at low exposures O^6-Me-Gua adducts are effectively repaired by O^6MT producing a higher *N*7-Me-Gua to O^6-Me-Gua ratio. As exposure increases, this O^6-Me-Gua specific DNA repair system is depleted and amounts of O^6-Me-Gua start to accumulate at a rate similar to their chemical formation. This may explain why early studies of adduct distribution with relative high exposures suggested that the adduct distribution *in vivo* is similar to the reaction of alkylating compounds with isolated DNA *in vitro* [8]. However as methodology improved, measurement at lower exposure concentrations were possible and differences in adduct profiles depending on exposures and tissue types were observed. Subsequent advancements in DNA repair research enabled correlation of specific DNA adduct profiles with repair capacities. In general, the dose response for *N*7-Me-Gua is linear, while it is sub-linear for O^6-Me-Gua in O^6MT repair proficient tissues. The slope of the upper portion of such a sub-linear dose response is similar to that of *N*7-Me-Gua and represents the chemical formation rate, since proportionately fewer adducts are removed by repair enzymes like O^6MT. At low exposures O^6-alkylguanine adducts are readily repaired in most tissues. Consequently, at higher exposures where DNA repair activity is saturated the number of DNA adducts formed increases per unit dose of carcinogen, producing a non-linear dose-response.

4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK), a tobacco-specific nitrosamine is a potent pulmonary carcinogen inducing lung tumors in experimental animals independent of route of exposure [83]. NNK is metabolically activated in several tissues by an P450-catalyzed hydroxylation of the methylene or methyl carbon adjacent to the *N*-nitroso group (α-hydroxylation, reviewed by Jalas et al. [91]). These hydroxylation reactions form unstable intermediates that spontaneously decompose to reactive diazonium ions, which can form DNA adducts including *N*7-guanine adducts, and formaldehyde (Figure 2)[92-100], as reviewed by Hecht [82]. The formation of NNK-derived *N*7-Me-Gua and O^6-Me-Gua has been observed in a number of tissues including placenta, esophagus, larynx, liver, and white blood cells (WBC) [101]. The dose-responses for *N*7-Me-Gua and O^6-Me-Gua in NNK-treated rat lung and liver tissues are similar in shape to the ones observed after DMN treatment discussed above. The dose-responses for *N*7-Me-Gua and O^6-Me-Gua are supra-linear in the lung and liver of NNK-treated rats due to saturation of the α-hydroxylation step in metabolic activation [102]. Furthermore, in lung the persistence of O^6-Me-Gua was greatest in Clara cells, the progenitor cells for nitrosamine-induced lung tumors [67,103,104]. NNK metabolism was shown to be greatest in Clara cells followed by alveolar macrophages, type II cells and minor in alveolar small cells (300, 220, 100, <10 mol/10^6cells/1h, respectively), demonstrating cell specific activation [69,69,103]. In addition, O^6MT activity was 2-fold greater in macrophages and Type II cells than in alveolar small cells or Clara cells [69,69,103]. Therefore, the Clara cell-specific accumulation of O^6-Me-Gua in rats was attributed to reduced O^6MT activity coupled with a high capacity for NNK activation.

2.3 Hydrazines

Similar to nitrosamines, hydrazine compounds have been studied both as potential anticancer drugs and as cancer-causing agents. Early studies of hydrazines, including hydrazine sulfate, were conducted and these compounds were found to induce tumors in laboratory animals [105-107]. Hydrazines require metabolic activation to exhibit their carcinogenic effects and experiments with rat liver microsomes suggested involvement of P450s [108]. Treatment of mice, rats or hamsters with hydrazines substantially increases the incidence of several tumor types [107]. When administered by gavage, 1,2-dimethylhydrazine (SDMH) increased the incidence of lung tumors in female mice. When administered in drinking water, SDMH induced high incidences of angiosarcomas in various organs and tumors of the kidneys, lungs, and liver in mice of both sexes [107]. The same route of administration induced liver carcinomas and angiosarcomas in rats.

Herron and Shank [109] investigated the time course of *N*7-Me-Gua in liver and kidney of rats over a 25-week period, during which the animals received 21 mg/kg SDMH s.c., once every week during the first 14 weeks. During the treatment period, *N*7-Me-Gua adducts accumulated in liver and kidney and then were below the limit of detection of 156 *N*7-Me-Gua/ 10^7 nnt six or 11 weeks post treatment. No adducts were found in non target tissues (lung or pancreas) [109]. Unfortunately, the limit of detection at these early studies was not sufficient to determine potential endogenous amounts of *N*7-Me-Gua, which are discussed in detail below. This study clearly demonstrated that metabolic activation, and subsequent adduct formation was tissue-specific, and that reactive hydrazine metabolites were not stable enough to diffuse to distant sites.

Surprisingly, in rats no adduct accumulation was found in colon, another target site for SDMH tumorigenesis [110-112]. In colon DNA, *N*7-Me-Gua and O^6-Me-Gua have the same half-life, ~30 h, which is much lower than the ~150 h determined in other tissues [29]. Therefore, the removal of *N*7-Me-Gua and other DNA adducts in colon is to a greater extent dependant on cell division and sloughing compared to liver, where DNA repair and spontaneous depurination are responsible for adduct removal [113]. O^6-Me-Gua accumulated in kidney, while in liver it was only detected after the first SDMH treatment, adding support to the inducibility of O^6MT in rat liver, but not in kidney, one of the main target organ of SDMH-induced tumorigenesis [89,114].

Furthermore, metabolic activation, detoxication, and DNA repair can be different among cell types. The first evidence for different DNA adduct profiles between cells within a target organ was demonstrated in rats exposed to single [32] or chronic [115] doses of SDMH. Swenberg and colleagues investigated the formation and persistence of *N*7-Me-Gua and O^6-Me-Gua in individual liver cell types. SDMH induces mainly malignant liver angiosarcoma [116-118]. Analysis of *N*7-Me-Gua in purified non-parenchymal cells (NPC), the origin of angiosarcomas, compared with hepatocytes demonstrated similar increases in both cell types, confirming even distribution of exposure between cell types. The corresponding amounts of O^6-Me-Gua were significantly different, with amounts in hepatocytes peaking one day after exposure and then declining to 1/30 of the initial amounts, while in NPC cells, amounts of O^6-Me-Gua steadily accumulated for 8 days [119]. The significant decline of O^6-Me-Gua in hepatocytes was due to both constitutive and an additional 4-fold enhancement of O^6MT activity during continuous exposure, an activity that is much lower and remained constant in NPC cells [119].

Summary—Even though O^6-Me-Gua was identified as the potentially promutagenic adduct in the examples described above, it would have been difficult to come to such a conclusion without normalization of the data to *N*7-Me-Gua. These and other studies with pro-carcinogens clearly established that metabolic activation of carcinogens and DNA alkylation can be species-, strain-, tissue-, and cell-type specific. In addition, evidence has been reported that

adduct formation rates and endogenous background levels could be age-dependent, and are modified by DNA repair. Consequently, these studies have greatly advanced our understanding in the origin and molecular mechanisms of tissue specificity of these model carcinogens. Unfortunately, as has been described above, the ratio of *N*7-guanine adducts to other DNA adducts (*e.g.*, O^6-guanine adducts) is not constant and is heavily dependent on time since exposure, dose, species, tissue, and cell type. Therefore, to use *N*7-guanine adducts as a surrogate biomarker for other DNA adducts, it is essential to have detailed knowledge of the rate of formation and persistence of *N*7-guanine adducts, and the corresponding adducts of interest.

2.4 Olefins: ethylene, propylene, butadiene, and their metabolites

Olefins are characterized by one or more double bonds, with ethylene (ET), propylene, and 1,3-butadiene (BD) being the best studied in respect to DNA adduct formation. BD and the ET metabolite, ethylene oxide (EO), have been classified as human carcinogens [120]. Occupational exposure to butadiene has been associated with increased risk for leukemia in workers exposed to BD in synthetic rubber production [121-123] and an increase in lymphohematopoietic cancers in BD monomer production workers that was not exposure related [124]. Olefins are mainly metabolized by P450s to the corresponding epoxides that are known to form DNA adducts, including *N*7-guanine adducts *via* S_N2 reactions (Figure 3). These olefin epoxides are more stable than the reactive metabolites discussed above (diazonium ion, hydrazine, *etc*.) and they circulate freely throughout the body. Consequently, DNA adducts are formed in similar amounts in target and non target tissues [68,125-127]). The main adducts of olefin epoxides are the corresponding *N*7-guanine adducts, and *N*3-adenine adducts in addition to other minor adducts. The relative reactivities of nucleophilic sites in DNA toward some olefin epoxides are shown in Table 1.

Ethylene and ethylene oxide—ET is metabolically activated by P450s to ethylene oxide (EO) and forms *N*7-hydroxyethyl-guanine (*N*7-HE-Gua) as its major DNA adduct. Inhalation studies with ET in mice and rats established supra-linear dose-responses over the exposure concentrations studied, suggesting saturation of the metabolic activation [128,129]. The metabolism of ET to EO in mice saturates at ~1000 ppm ET [130,131], producing in liver ~3.5 *N*7-HE-Gua/ 10^7 nnt (Table 2,Figure 4) [33,132,133]. In contrast, a linear dose-response for *N*7-HE-Gua has been found in experiments with mice or rats exposed to EO over a wide range of exposures (Figure 4)[133,134]. The molecular dose of *N*7-HE-Gua can be orders of magnitude greater for exposures to EO than can ever be achieved by ET. In subacute or chronic exposures to EO, *N*7-HE-Gua adducts increase daily until they reach a steady state after 7-10 days [33]. This supra-linear response over time is attributed to the chemical instability of *N*7-HE-Gua. At steady state, the number of *N*7-HE-Gua adducts formed is equal to the number of adducts lost due chemical depurination or cell death.

All animals and humans are constantly exposed to ET and EO produced by numerous endogenous metabolic processes and by conversion of ET to EO by the liver [135,136]. Additional EO exposures are from numerous food products that can contain up to <0.05 to 1800 μg ET/g [137]. The endogenous formation and steady exposure *via* the diet produces a background level of *N*7-HE-Gua that has been observed in mice, rats, and humans [128, 138-143]. Yong *et al*. reported *N*7-HE-Gua in granulocytes from humans that were not exposed to known sources of ET or EO [143]. The presence of endogenous EO exposure in humans has been confirmed by detection of hydroxyethyl-valine hemoglobin adducts, another EO-specific biomarker [144,145]. Endogenous or background amounts in mice and rats were in the range of 1-18 *N*7-HE-Gua adducts/10^7 nnt [132,133,146-148]. The existence of endogenous *N*7-guanine adducts has to be accounted for in mechanism-based risk assessments and evaluations of low exposures [27].

Propylene and propylene oxide—Propylene is metabolically activated by P450s to propylene oxide (PO) and forms *N*7-hydroxypropyl-guanine (*N*7-HP-Gua). The formation of *N*7-HP-Gua in rat liver saturates at exposures greater than 2000 ppm propylene and produces about 6.5 adducts /10^7 nnt (Figure 4) [149]. In contrast, exposures to the activated oxide, PO, produces almost 200-fold greater amounts of *N*7-HP-Gua and the dose-response is linear (Figure 4). Comparing the adduct amounts formed after propylene and PO exposures, it can be estimated that a maximum of 0.5% of the propylene dose is activated to PO and associated with DNA adduct formation. The fact that the adduct formation appears linear for PO-derived *N*7-HP-Gua and that exposures to PO produce much higher amounts of these adducts demonstrates that adduct formation is not limited by available binding sites in DNA or induction of specific DNA repair systems. Therefore, the saturation observed with propylene is due to saturation of the metabolic activation and/or induction of detoxification pathways. Similar data were obtained from a variety of chemicals and are summarized in Table 2.

Butadiene and butadiene-derived epoxides—1,3-Butadiene (BD) is an olefin of special interest because it is metabolized to several reactive epoxides (Figure 3)[150]. All BD-derived epoxides are known to form DNA and protein adducts. Althought the different oxidation reactions are catalyzed by the same enzymes, mainly by P450 2E1, 2A6 and 3A4, the dose-responses of their internal formation are vastly different in mice, rats, and humans [151,152]. BD is first oxidized to 1,2-epoxy-3-butene (EB), a metabolite known to form 2-hydroxy-3-butenyl DNA and protein adducts. To date, the *N*7-(2-hydroxy-3-butenyl)-guanine and *N*7-(1-hydroxy-3-butenyl)-guanine (*N*7-HB-Gua)* are the only EB-specific *N*7-guanine adducts found in mice and rats exposed to BD (Figure 3)[68]. The dose-response for *N*7-HB-Gua has been shown to be linear in mice and rats from the lowest exposure studied (20 ppm BD 4 weeks) to high exposures known to induce tumorigenesis [68]. The presence of a minor adduct, *N*1-HB-Ade, has been reported in rat liver after 5 days of exposure to 300 ppm BD [153] and its formation was about 3-fold lower than the *N*7-HB-Gua.

In vitro formation of *N*3-HB-adenine, N^6-HB-adenine, and *N*1-HB-inosine adducts have been identified, but their existence *in vivo* has not been shown [154,155]. Of these theoretical adducts, *N*1-HB-inosine is of special interest, since it has the highest mutagenic potency (>95% per replication cycle) [156,157]. The mutagenic potencies were significantly lower (<1%) for N^2-HB-guanine and N^6-HB-adenine [158]. Unfortunately, the *N*7-HB-Gua adduct is not suitable for site-directed mutagenesis studies because of its chemical instability that leads to spontaneous depurination. Therefore, the specific mutagenic potency of *N*7-HB-Gua remains to be assessed, although mutagenic potency may be very low given that *N*7-guanine adducts are relative unstable and do not participate in hydrogen bonding in the DNA double helix [2].

EB can undergo a second oxidation reaction, catalized by P450s 2E1, 2C9, and 2A6, producing the 1,2;3,4-diepoxybutane (DEB) [159], which is a bi-functional carcinogen that can form DNA-DNA [160-162] and DNA-protein crosslinks (Figure 3)[163,164]. *In vivo*, the presence of *N*7-guanine-*N*7-guanine [1,4-bis(guan-7-yl)-2,3-butanediol (bis*N*7-Gua-BD-diol)] and *N*7-guanine-*N*1-adenine [1-(guan-7-yl)-4-(aden-1-yl)-2,3-butanediol (*N*7-Gua-*N*1-Ade-BD-diol)] were observed in liver and lung of mice exposed by inhalation to 625 ppm BD [160, 161]. The bis*N*7-Gua-BD-diol is a weak mutagen, producing miscoding in less than 1% of all replication cycles, compared to the more mutagenic N^6,N^6-adenine intrastrand cross-links, which produce miscoding in 8% of the replication cycles, [156,157]. The dose-responses of bis*N*7-Gua-BD-diol and *N*7-Gua-*N*1-Ade-BD-diol cross links were recently reported in mice and rats [165]. It was shown that mice form much more DEB than rats and that adduct formation was higher in lung and liver compared to kidney, brain and thymus. The tissue difference

*The abbreviation N7-HB-Gua will be used for both the N7-(2-hydroxy-3-butenyl)-guanine and N7-(1-hydroxy-3-butenyl)-guanine (N7-HB-Gua).

appears to be due to the high reactivity of the bi-functional DEB compared to the mono-functional EB or EB-diol and suggests DEB formation in liver and lung tissue. Further, formation of DEB seemed to saturate in rats at exposures above 62.6 ppm BD and showed a decrease in slope in mice at exposures above 200 ppm. This dose-response for DEB derived adducts have been confirmed by the analysis of *N*,*N*-(2,3-dihydroxy-1,4-butadiyl)-valine (*pyr*-Val), the corresponding DEB-specific globin adduct, in the same animals (Georgieva et al in preparation).

The epoxides EB and DEB can be hydrolyzed by epoxide hydrolase (EH) producing 3-butene-1,2-diol (BD-diol) and 1,2-epoxy-3,4-butanediol (EB-diol), respectively [151, 166-171]. The latter, EB-diol, can also be formed by a second oxidation of BD-diol [167, 171]. Analysis of the EB-diol-derived *N*7-(trihydroxybutanyl)-guanine (*N*7-THB-Gua) adducts demonstrated that EB-diol is the main BD-derived epoxide in mice and rats (Figure 3)[68]. Most interestingly, while the dose-response for *N*7-HB-Gua (EB-derived) is linear, the dose-response for *N*7-THB-Gua (EB-Diol derived) shows saturation at exposures greater then 62.5 ppm BD in rats after 20 days of exposure, and its slope of formation is reduced with higher exposures in mice [68]. In addition, using *N*7-HB-Gua and *N*7-THB-Gua, it was shown that mice form much more EB and EB-diol than rats, a finding that is consistent with the higher susceptibility of mice to BD induced tumorigenesis.

BD species-dependent tumorigenesis is attributed to species-specific differences in BD metabolism. The formation of *N*7-guanine adducts support the hypothesis that mice are more susceptible because they are more efficient at BD oxidation. Molecular modeling of P450 2E1 also suggested significant species differences between mouse, rat, and human in BD oxidation [172]. The species differences in formation of *N*7-HB-Gua, DEB-derived cross-link adducts and *N*7-THB-Gua has been confirmed using the corresponding globin adducts hydroxybutenly-valine (HB-Val), *pyr*-Val and trihydroxy-butanly–valine (THB-Val), suggesting that the corresponding hemoglobin adducts are a good biomarker for formation of these reactive metabolites [173,174]. Lastly it was shown that EB can bind to human P450 2E1 itself [175], and may alter catalytic activity in a similar fashion as the mechanism-based inhibition by other P450 substrates including ET [176-186]. Thus, monitoring *N*7-HB-Gua may provide a biomarker for functional changes in the metabolic pathways, possibly due to alkylating inactivation of important enzymes.

In summary, the studies with olefins described above clearly demonstrate that direct alkylating agents produce a linear dose-response for *N*7-guanine adducts vs dose, confirming the validity of *N*7-guanine adducts as a biomarker for internal dose. In contrast, adducts resulting from metabolites of metabolically activated compounds show strong species differences and usually exhibit supra-linear dose-response curves. This is primarily due to saturation of metabolic activation or changes in detoxification pathways, which means that the *N*7-guanine adducts are better dosimeters for internal dose than administered dose, especially if quantified in tissues of interest. In chronic exposure situations, steady state levels are typically reached after 7-10 days for both olefins and their epoxide metabolites.

3 Formation of *N*7-guanine adducts in human specimens

Despite the ubiquitous nature of *N*7-guanine adducts, their application as a biomarker of exposure in larger molecular epidemiology studies is not common practice. A review of the literature demonstrated limited numbers of studies using *N*7-guanine adducts as biomarkers for exposure to environmental or occupational pollutants. Furthermore, reported data are not extensive and mostly contain small numbers of individuals per group.

Similar to the data from animal studies, the presence of *N*7-Me-Gua and other *N*7-guanine adducts has been demonstrated ubiquitously in DNA from humans not known to be exposed

to alkylating agents (Table 3) [146,187-196]. Most of these reports investigated DNA from blood samples; either white blood cells (WBC) or individual blood cell types, and measured *N*7-Me-Gua by ^{32}P-postlabeling or HPLC-ECD. While this seems to be sufficient evidence for the presence of *N*7-Me-Gua from endogenous sources in human DNA, it needs to be mentioned that the numbers of subjects per group in most of these studies were relatively small (≤20). The amount of *N*7-Me-Gua in controls, representing endogenous or background adducts, were about half of the amounts reported for the exposed groups, which were primarily smokers. Consequently, the statistical power of these studies is reduced because of the small number of subjects and the limited difference between controls and exposed indviduals. Furthermore, it is not possible to differentiate endogenous adducts from background adducts, *e.g.*, formed from potential environmental sources due to their indistinguishable chemical identity. Interestingly, *N*7-Me-Gua amounts were 3-fold and 5-fold higher in tumor tissue compared to normal tissues from cervical or bladder tissues, respectively [187,194]. In contrast, N7-Me-Gua amounts were lower in colon tumor DNA compared to normal colon tissue [192]. The amounts of *N*7-Me-Gua in all controls (representing endogenous background amounts) range from 0.8 to 13.5 adducts/10^7 nnt compared to 3.9 to 23.6 adducts/10^7 nnt in the exposed subjects from the corresponding study groups. Evaluation of *N*7-Me-Gua amounts in lymphocyte DNA and solid tissues suggest slightly higher amounts in solid tissues, however sample numbers are small (Table 3).

Wu *et al.* reported mean endogenous amounts of 4.8 ± 3.1 *N*7-HE-Gua adducts / 10^7 nnt in lymphocytes (n=23) of unexposed humans [128]. More recently, Yong *et al.* [143] demonstrated the presence of *N*7-HE-Gua in granulocytes of hospital workers not known to be exposed to sources of ET or EO [143]. The mean amount of *N*7-HE-Gua was 9.6 ± 5.8 adducts/ 10^7 nnt in subjects categorized as unexposed controls. The range of these amounts is similar as reported previously for five control subjects [141]. These *N*7-HE-Gua background amounts are believed to stem from EO that forms endogenously from ET derived from environmental exposures such as vegetation, urban air, smoking, and various endogenous metabolic processes [137,197-199]. Tompkins *et al.* report in this special issue evidence of increased *N*7-HE-Gua in rat tissue due to oxidative stress [200]. H_2O_2 may catalize the formation of ET from methionine, similar to the ET production in fruits and vegetables [201, 202]. The confirmed presence of background DNA alkylation (*N*7-Me-Gua and *N*7-HE-Gua) from environmental and endogenous sources in humans is relevant for consideration in risk assessments used to make regulatory decisions. No evidence for endogenous *N*7-HP-Gua and *N*7-THB-Gua have been reported, although presence of hydroxypropyl-valine (HP-Val) and THB-Val, the corresponding PO- and EB-diol-specific globin adducts, have been reported in subjects or animals not exposed to known sources of propylene, BD or their epoxide metabolites, PO and EB-diol, respectively [126,203,204]. This may suggest that additional unknown endogenous *N*7-guanine adducts may exist that need to be considered.

4 Evidence for mutations resulting from *N*7-guanine adducts

4.1 *N*7-Me-Gua and *N*7-Et-Gua and mutagenesis in mammalian cells

Early efforts aimed to compare DNA alkylation with mutation frequency (MF) and mutation spectra to identify adducts involved in mutagenesis. Beranek *et al.* reported a good correlation between DNA methylation (*N*7-Me-Gua and O^6-Me-Gua) and mutation frequency (MF) in the *HPRT* gene in CHO cells after treatment with MMS or MNU [205]. In contrast, the formation of *N*7-Et-Gua did not correlate with mutation frequency in *HPRT* or Na-K-ATPase genes in CHO cells treated with diethylsulfate (DES), EMS, or ENU [206]. The formation of *N*7-Et-Gua was linear with exposures for each ethylating agent, but the slopes were significantly different. In contrast to the *N*7-Et-Gua, the amounts of O^6-Et-Gua adducts induced by all three ethylating agents correlated with MF in the *HPRT* gene, while the MF in the Na-K-ATPase gene correlated only with EMS and ENU, and not with DES [206]. This suggests that such

correlation studies may be inadequate to analyze multi-component phenomena like mutagenesis, and that DES induces mutations by a mechanism not involving *N*7-Et-Gua adducts.

Methyl methanesulfonate (MMS) predominantly produces *N*7-Me-Gua (~92%) in DNA and a very low percentage of mutagenic O^6-Me-Gua (~0.3%) [21]. In Chinese hamster ovary (CHO) cells exposed to MMS at 0.75 mM for 1 hour, the majority of *HPRT* mutations were GC to AT transitions [207], with no increase in other types of mutations. This type of mutation is also found in other Chinese hamster cell lines following exposure to MMS either at 2 mM for 30 minutes or at 1 mM for 1 hour [208,209]. In these three studies, MMS appears to introduce 750 to 1000 *N7*-Me-Gua/10^7 nnt, 0.8 to 1.0 *N*3-Me-dAde/10^7 nnt, and 0.027-0.036 O^6-Me-Gua/ 10^7 nnt roughly estimated from our data and from the ratio of *N*7-Me-Gua/*N*3-Me-Ade/O^6-Me-Gua = 83/8.7/0.3 [21]. It was concluded that this specific GC to AT transition is most likely caused by mispairing of O^6-Me-Gua with thymine during replication and cell division [210,211]. These results strongly suggest that AP sites likely formed by spontaneous depurination of *N*7-Me-Gua do not contribute to the induction of *HPRT* mutations in these MMS-exposed cells. Interestingly, preliminary results from our lab show that an imbalance of base excision repair (BER) repair in CHO AA8 cells may start at exposure to 1.9 mM MMS for 1 hour as determined by the extent of depletion of intracellular NAD(P)H in cells (Figure 5) (Pachkowski, unpublished data). Therefore, if the *N*7-Me-Gua are 1000 lesions/10^7 nnt or less and *N*3-Me-Ade are 100 lesions/10^7 nnt or less, the increase in *HPRT* mutations in cells exposed to MMS can all be accounted for by induction of O^6-Me-Gua (0.036 lesions/10^7 nnt or less) and are not due to formation of AP sites as BER intermediates.

4.2 *N*7-HE-Gua and *N*7-HP-Gua and mutagenesis

The formation of *N*7-HP-Gua saturates at levels of 6.5 adducts /10^7 nnt in rats after 20 days of exposure to propylene (Table 2, Figure 4) [149]. This amount is 15-fold lower than the molecular dose associated with induction of mutations in *Drosophila* [212]. In that study, male *Drosophila* exposed to PO for 24 h did not show a significant increase in mutations until the internal dose, measured as *N*7-HP-Gua adducts, reached approximately 100 adducts /10^7 nnt. Since no other adducts were measured, these data do not demonstrate that *N*7-HP-Gua was the causal adduct. Together, these reports provide mechanistic evidence that propylene is not mutagenic. Pottenger *et al.* [149] demonstrated that metabolic activation of propylene does not produce sufficient numbers of DNA adducts in mice or rats, compared to that associated with mutagenesis in *Drosophila* [212]. Furthermore, the *Drosophila* system most likely over estimates the mutagenic potency of PO since cell proliferation is much faster in the *Drosophila* gonadocytes, leaving less time for DNA repair compared to mammalian systems.

The range of *N*7-guanine adducts of 0.8-23 adducts /10^7 nnt in humans, summarized in Table 3, are significantly below the *N*7-HP-Gua adducts shown to correlate with mutations in *Drosophila*. In contrast, significant increases in mutations in Drosophila after EO exposure were accompanied by 30 *N*7-HE-Gua/ 10^7 nnt. However NER repair decreased the EO-induced mutation response in *Drosophila*, suggesting involvement of adducts other then *N*7-HE-Gua in mutagenesis [212]. This adduct amount is close to the amounts observed in hospital workers exposed to EO as a sterilant and only 3 to 6-times higher than the background level of endogenous *N*7-HE-Gua [143]. These data may suggest that the large background of endogenous DNA adducts, derived from endogenous metabolites arising from oxidative stress and endogenous ET may significantly contribute to background mutagenesis [200,213]. This is an area that clearly needs additional research.

4.3 AP sites derived from *N*7-guanine adducts

Apurinic sites (AP) are the most common form of endogenous DNA damage and are the product of spontaneous depurination, oxidative damage, and the result of DNA repair. The notion that *N*7-guanine adducts convert to AP sites and subsequently cause mutations was investigated by Rusyn *et al.* [129]. It was demonstrated that AP sites are not increased following repeated exposure to ET or EO; the presence of *N*7-HE-Gua indicates that AP sites would be formed by spontaneous depurinations, so they must be efficiently repaired so that they do not cause any increase over the endogenous level [129]. In addition, the *HPRT* mutation frequency in mice and rats, and micronuclei in polychromatic or normochromatic erythrocytes (MNPCE) in rats, were not increased after repeated exposures to 3000 ppm ET, while the *N*7-HE-Gua adducts significantly increased over endogenous levels and their formation saturated at exposures of 3000 ppm ET. This exposure resulted in 6 adducts /10^7 nnt (Table 2) [33]. In contrast, exposures of 100 ppm EO significantly increased *HPRT* mutation frequency and MNPCE [33,214], but still did not result in accumulation of AP sites [129]. The number of AP sites ranged from 30 to 70 AP sites /10^7 nnt for controls, ET-, and EO-exposed rats, while the number of *N*7-HE-Gua increased more than 10-fold from ~6 to 64 /10^7 nnt in rats exposed to 0 or 100 ppm EO for 4 weeks, respectively [129]. The authors concluded that the mutagenesis from EO exposures most likely resulted from minor promutagenic adducts, rather than AP sites or *N*7-HE-Gua. Measurement of AP sites after neutral thermal hydrolysis of DNA confirmed that EO produced heat labile lesions as a result of enhanced depurination. Heat-treated spleen DNA had a 5-fold increase in AP sites after EO exposure [129].

Similarly, PO exposures did not increase AP sites in nasal epithelium, the target tissue for PO carcinogenesis, despite the high internal dose of *N*7-HP-Gua, with up to ~1000 *N*7-HP-Gua / 10^7 nnt in rats exposed to 500 ppm PO for 4 weeks [215]. While the number of adducts was much lower than that found after high PO exposures, 4 week inhalation exposures to 10,000 ppm propylene did not increase the mutation frequency in the *HPRT* gene in spleenocytes [149].

AP sites are also intermediates of BER, however, formation from enzymatic removal of *N*7-guanine adducts is minimal, compared to those arising from spontaneous depurination. This area is not fully understood, however, enzymatic removal has been shown to reduce the half-lives of some *N*7-guanine adducts to minutes instead of hours [216-218]. The bacterial enzyme AlkA has been shown to remove a wide variety of damaged purine and pyrimidine bases, including *N*7-Me-Gua [219-221]. Mammalian cells do not possess a homologue of AlkA, but AAG, a member of the *N*-methylpurine DNA glycosylase family, removes a broad spectrum of modified purines from DNA including *N*7-Me-Gua [222,223]. Additional studies on knockout animals will be needed to demonstrate that DNA repair plays a significant role in removal of *N*7-guanine adducts, compared to chemical depurination.

4.4 Mutations induced by BER intermediates in mammalian cells

To characterize the mutagenic potential of AP sites, a BER intermediate in mammalian cells, an SV-40-derived shuttle vector with a single lesion of either an abasic (AP) site or 5′-deoxyribose-5-phosphate (5′dRp: 5′-cleaved AP sites), at a defined position, was transfected into monkey kidney COS7 cells [224]. The mutation spectrum revealed that preferential incorporation opposite the AP sites is dA (48%) > dC (39%) > dG (13%) ≫ dT (none). Thus, if either dA or dC is incorporated opposite the AP sites derived from depurination of *N*7-guanine adducts, GC->TA transversions are expected as the primary mutation induced by AP sites. In addition, a small proportion (16%) of deletions was also observed [224]. During BER, the AP sites are converted into single strand breaks (SSB) by AP endonuclease 1, which leads to the formation of 5′dRp and 3′ hydroxyl termini. In the same vector system, the mutation analysis showed that preferential incorporation opposite the 5′-dRp was dA (46%) > dG (41%)

≫ dC (13%) ≫ dT (none). As with AP sites, a small proportion (9%) of deletions was observed. Therefore, *N*7-guanine adducts are expected to cause G->T transversions, G->C transversions, and deletions if they result from products of BER intermediates 5′dRp and AP sites. In contrast, the nitrosamine DMN and ENU induce mainly G->A transition mutations in the BigBlue transgenenic mice model [225-228]. In CHO cells, MMS primarily increases GC to AT transitions in the HPRT gene [207]. In splenic T-lymphocytes of mice, EO induced four A:T transversions, three A:T transitions, two G:C transversions, and two G:C transitions [229]. In *in vitro* and *in vivo* test systems, BD and its metabolites, EB, DEB, and EB-diol induce predominantly deletions and base substitutions at A:T [230-232]. Consequently, the mutation spectra are quite different for compounds known to form *N*7-guanine adducts that can convert to AP sites, than the mutation spectrum produced by AP sites.

4.5 Ring-opened formamidopyrimidine adducts (FAPy adducts)

*N*7-guanine adducts are susceptible to attack by hydroxide on the C8 carbon and subsequent ring opening to form 5-*N*-alkyl-2,6,-diamino-4-hydroxyformamidopyrimidine (Alkyl-FAPy) [233-241]. Early studies [233,239] proposed placement of a formyl group (-CHO) on the *N*7-position and this has been confirmed by NMR examination of 5-*N*-methyl-2,6,-diamino-4-hydroxyformamidopyrimidine (Me-FAPy) and 5-*N*-(9-hydroxyaflatoxin)-2,6,-diamino-4-hydroxyformamidopyrimidine (AFB_1-FAPy) [242,243]. During the ring opening, the negative charge on the N9-position is delocalized to an α-β-unsaturated carbonyl by the pyrimidine ring, subsequently stabilizing the glycosidic bond. This has important biological implications since, unlike the *N*7-guanine adduct, the Alkyl-FAPy will not spontaneously depurinate and be lost from DNA. In fact it has been shown that the Me-FAPy was more persistent in rat bladder epithelium than O^6-Me-Gua after 21 days and accounted for approximately 72% of the total MNU-derived adducts [244]. In rat liver Me-FAPy was the main persistent adduct after DMN and SDMH treatment [245]. There is an urgent need for more data on the amount of Me-FAPy and other FAPy adducts in cells and tissues in exposed and control animals.

For example, aflatoxin, one of the most potent liver carcinogens, is metabolically activated to the 8,9-epoxide which forms primarily *N*7-AFB_1-Gua [246-252]. The *N*7-AFB_1-Gua converts to secondary lesions including AP sites and AFB_1-FAPy. Compared to *N*7-AFB_1-Gua, AFB_1-FAPy is highly persistent in rat liver DNA, reaching maximum amounts 2 weeks after exposure [249]. While the structures of *N*7-AFB_1-Gua and AFB_1-FAPy are similar, they alter the secondary DNA structure differently [242,253]. The increased chemical stability (persistence) and altered secondary DNA structure are believed to be responsible for the much greater mutagenicity of AFB_1-FAPy compared to *N*7-AFB_1-Gua. In fact, *N*7-AFB_1-Gua and/or AFB_1-FAPy cause primarily G to T mutations, consistent with the observed G to T mutations in codon 249 of the p53 tumor suppressor gene in 50% of hepatocellular carcinomas and in aflatoxin-treated human hepatocytes cultures [254,255]. Additionally, aflatoxin-induced G to T mutations in the *ras* oncogene are assumed to be important in tumor progression [256-258]. Although both *N*7-AFB_1-Gua and AFB_1-FAPy cause the types of mutation consistent with the ones observed in aflatoxin-exposed biological systems, the MF is 6-fold lower (4% vs 32%) for *N*7-AFB_1-Gua compared to AFB_1-Fapy, respectively [259,260]. Whether the *N*7-AFB_1-Gua converted to AFB_1-FAPy during the mutation assay was not determined, however, if this were the case, the mutagenic potency for the *N*7-AFB_1-Gua would be even less, making the AFB_1-FAPy even more important. This suggests that the AFB_1-FAPy, and potentially other ring-opened *N*7-guanine adducts, may be the ultimate lesions responsible for mutagenesis and genotoxicity of aflatoxin and other carcinogens. Unfortunately, there are no data on the formation and persistence of other FAPy adducts and future investigations of *N*7-guanine adducts should include studies on the ring-opened FAPy derivates.

5 Conclusions

After decades of research on *N*7-guanine adducts in animals and humans, it has become clear that specific *N*7-guanine adducts are excellent biomarkers for internal exposure when they are determined in tissue DNA. In contrast, *N*7-guanine adducts that can be formed from endogenous or background sources are less reliable for estimating low external exposures. While the presence of *N*7-guanine adducts clearly demonstrate exposure to the tissues or cells, subsequent interpretations and conclusions need to consider that the value of this endpoint is complicated by endogenous and background formation, and can vary according to species, age, duration of exposure, and tissue. From our review of the literature, we did not find any evidence that *N*7-guanine adducts can be used beyond confirmation of exposure to the target tissue and demonstration of the molecular dose. This is especially important for translation to human studies, since most studies consist of a single time point analysis of an individual adduct. If the goal is to confirm exposure or identify factors that increase endogenous formation, analysis of N7-guanine adducts in DNA may be sufficient. Consequently, values of *N*7-guanine adducts in human urine (not reviewed herein) may be sufficient to identify exposures or factors increasing endogenous formation between population groups, even if no information about the site of formation will be available [246,261,262]. Importantly, a correlation with other endpoints like chromosome aberrations or mutation frequency cannot be expected to be definitive, since such biomarkers of effect are not chemical-specific and are products of complex biological processes that may only partly (or even not at all) involve *N*7-guanine adducts.

The main challenge in studying the mutagenic potency of *N*7-guanine adducts is that their chemical instability has prevented systematic investigation of the lesions in site-directed mutagenesis studies. In fact, oligodeoxynucleotides containing *N*7-Me-Gua have been prepared, using DNA polymerase and structural analysis by NMR revealed no disturbance of the b-DNA helix [263]. However, since *N*7-guanine adducts are not considered stable enough to undergo site-directed mutagenesis studies, an alternative would be to stabilize the glycosidic bond by either substitution of guanine with 2-amino-8,9-dihydro-1H-purin-6(7H)-one or by incorporation of a fluorine at the 2′-position of the 2′deoxyribose moiety as recently reported by Lee et al. [264]. The ring opened *N*7-Me-FAPy adduct of N7-Me-Gua have been shown to block DNA polymerase *in vitro* [265,266] but its relevance *in vivo* needs to be determined. Finally, the formation of N7-guanine adducts is often accompanied by formation of other adducts (*e.g.*, O^6-Me-Gua) that are known to be highly mutagenic lesions. Mechanistically, the lack of mutagenicity is linked to the fact that the *N*7-position does not participate in hydrogen bonding in the DNA double helix, unlike the *N*1, N^2, or O^6-positions of Gua [2].

It is noteworthy that covalent binding to DNA and the ability to form *N*7-guanine adducts alone has long been considered evidence of genotoxicity for several drugs and chemicals [267]. Conversely, *N*7-guanine adducts easily depurinate to produce AP sites, however, these lesions are the most common endogenous DNA damage, and their number does not appear to increase even with extremely high exposures to alkylating agents. Therefore, one needs to be cautious in classifying compounds and drugs based on formation of *N*7-guanine adducts alone. This becomes very important for risk assessment of low chronic exposures, when data are limited to high and single dose exposures. The default is to assume a linear dose-response to zero and subsequent extrapolation of the DNA damage data from high to low dose. However, examples have been reported that show that at low exposures, biological processes like mutagenesis reach a background that is much different in slope than *N*7-guanine adducts [27,200]. It was suggested that these background mutations are driven by endogenous processes rather than by external exposure. For example, using [^{13}C]MMS for cell treatment and mass spectrometry quantition of [^{13}C]*N*7-Me-Gua and *N*7-Me-Gua, Swenberg *et al.* demonstrated that the adduct formation by MMS from exogenous sources is linear towards zero, while the dose-response

for mutation frequency follows a “hockeystick” threshold dose-response (Figure 6)[27]. Similar data have also been presented for EO, EMS, and acrylamide [27,200,268]. Based on the information presented in Section 4, it is very unlikely that exposures resulting in elevated amounts of *N*7-guanine adducts are sufficient to induce mutations, since pro-mutagenic lesions, such as O^6-guanine adducts, will also increase and are expected to drive mutagenesis.

A careful review of the literature related to *N*7-guanine adducts revealed that there is little evidence that *N*7-guanine adducts cause mutations. In addition, there is mounting evidence that they do not cause mutations, since they do not participate in hydrogen bonding and easily depurinate. In addition, formation of *N*7-guanine adducts is often accompanied by the formation of other adducts, known to be mutagenic, and therefore it is difficult to credit mutation events to *N*7-guanine adducts. As discussed in detail before, the relative ratio between *N*7-guanine adducts and other adducts (*e.g.*, O^6-alkylguanine) is dependent upon the chemical and modified by species, strain, tissue, or cell types, differences in activation, and differences in DNA-repair. Consequently, the formation of *N*7-guanine adducts cannot be used in isolation as a quantitative biomarker for promutagenic DNA lesions, mutagenic response or as a surrogate for other biological processes.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

AFB_1-FAPy
5-*N*-(9-hydroxyaflatoxin)-2,6,-diamino-4-hydroxyformamidopyrimidine

Alkyl-FAPy
5-*N*-alkyl-2,6,-diamino-4-hydroxyformamidopyrimidine

AGE
allyl glycidyl ether

AP-sites
apurinic sites

BER
base excision repair

bis*N*7-Gua-BD-diol
1,4-bis(guan-7-yl)-2,3-butanediol

DEB
1,2;3,4-diepoxybutane

DEN
diethylnitrosamine

DES
diethylsulfate

EB
1,2-epoxy-3-butene

EB-diol
1,2-epoxy-3,4-butanediol

ECH
epichlorohydrin

EMS
ethylmethanesulfonate

ENU
ethylnitrosourea

EO
ethylene oxide

ET
ethylene

HENU
1-(2-hydroxyethyl)-1-nitrosourea

HPLC-ECD
High pressure liquid chromatography with electro chemical detection

ISB
Immunoslot blot

KC
Kupffer cells

LC-MS/MS
Liquid chromatography tandem mass spectrometry

Me-FAPy
5-*N*-methyl-2,6,-diamino-4-hydroxyformamidopyrimidine

MF
mutation frequency

MMS
methylmethanesulfonate

MN
micronucleus

MNU
N-methyl-*N*-nitrosourea

***N*7-AFB$_1$-Gua**
*N*7-(9-hydroxyaflatoxin)-guanine

***N*7-Et-Gua**
*N*7-ethyl-guanine

***N*7-Gua-*N*1-Ade-BD-diol**
1-(guan-7-yl)-4-(aden-1-yl)-2,3-butanediol

***N*7-HB-Gua**
*N*7-hydroxybutenly-guanine

***N*7-HE-Gua**
*N*7-hydroxyethyl-guanine

***N*7-HMGSH-Gua**
S-[1-(hydroxymethyl)-2-(*N*7-guanyl)ethyl]-glutathione

***N*7-HP-Gua**
*N*7-hydroxypropyl-guanine

***N*7-Me-Gua**
*N*7-methyl-guanine

***N*7-THB-Gua**
*N*7-trihydroxybutanly-guanine

nnt
normal nucleotides

NPC
non-parenchymal cells

NS
non smokers

***O*^{2}-Et-Thy**
O^2-ethyl-thymidine

***O*^{4}-Et-Thy**
O^4-ethyl-thymidine

***O*^{6}-Et-Gua**
O^6-ethyl-guanine

***O*^{6}-Me-Gua**
O^6-methyl-guanine

***O*^{6}MT**
O^6-methylguanine DNA-methyltransferase

^{32}P
^{32}P-postlabeling

^{32}P-AEC
^{32}P-postlabeling anion exchange chromatography

PO
propylene oxide

S
smokers

SDMH
1,2-dimethylhydrazine

SEC
sinusoidal endothelia cells

SO
styrene oxide

SSB
single strand breaks

TCP
1,2,3-Trichloropropane

THB-Val
trihydroxybutanly–valine

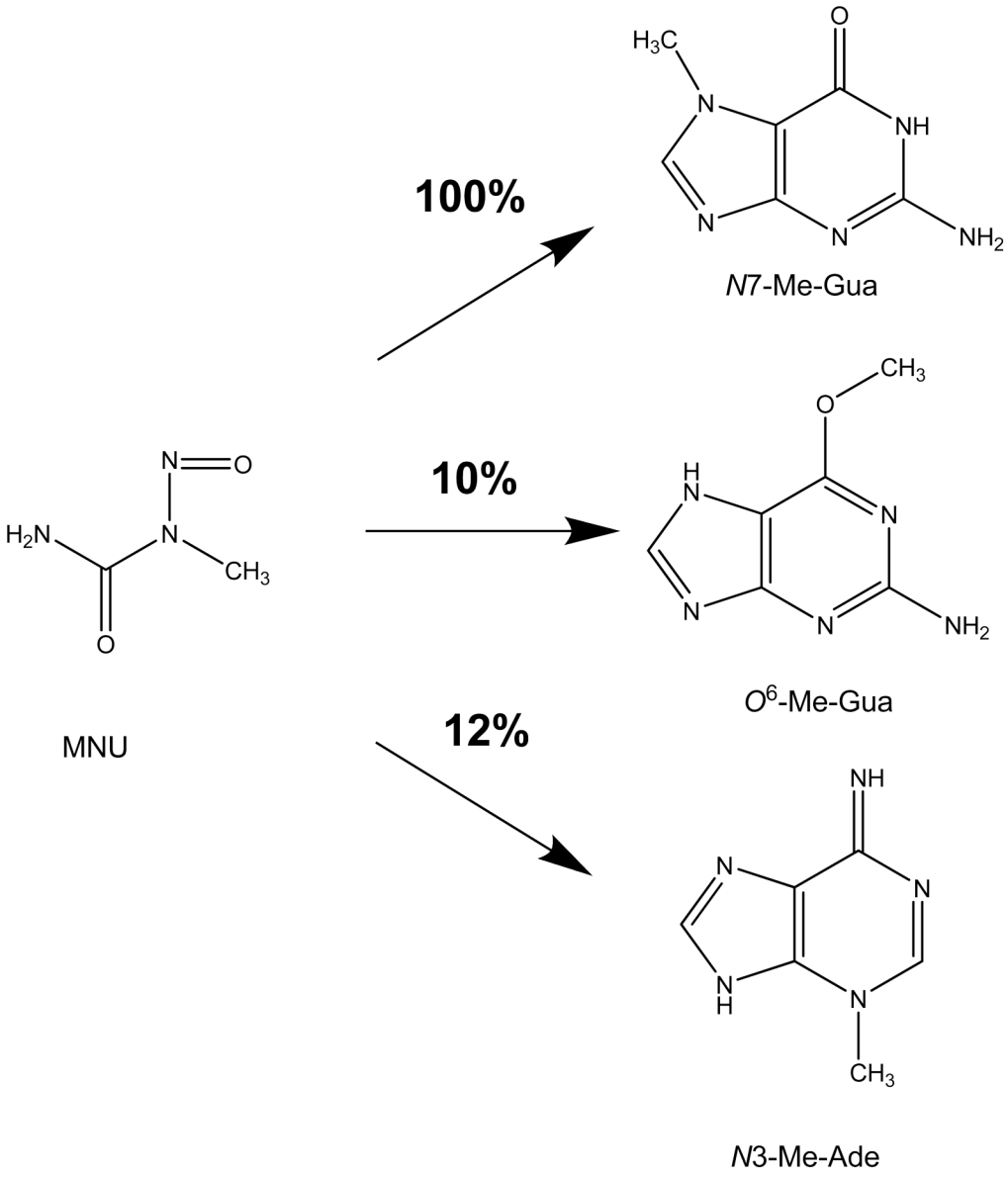

Figure 1.
Guanine and adenine methylation by MNU relative to *N*7-Me-Gua formation (100%).

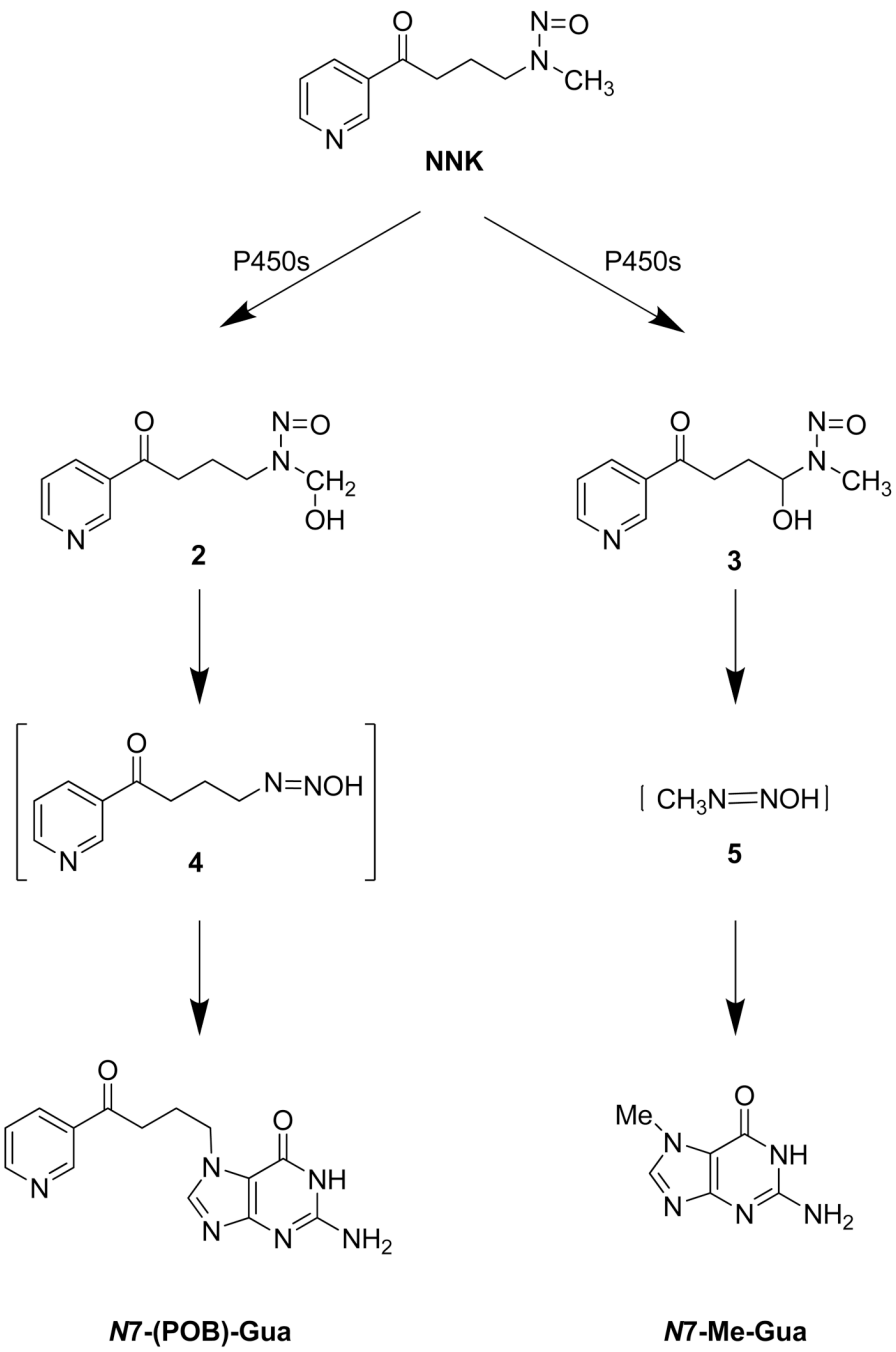

Figure 2.

Formation of *N*7-guanine adducts form diazonium ion metabolites of NNK. NNK is metabolized by P450–catalyzed α-hydroxylation, producing hydroxymethyl NNK (2) and α-methylenehydroxy NNK (3), which spontaneously decompose to their corresponding diazonium ions (4,5), and keto aldehyde and formaldehyde, respectively. The highly reactive diazonium ions (4,5) subsequently form DNA adducts including *N*7-guanine adducts [82].

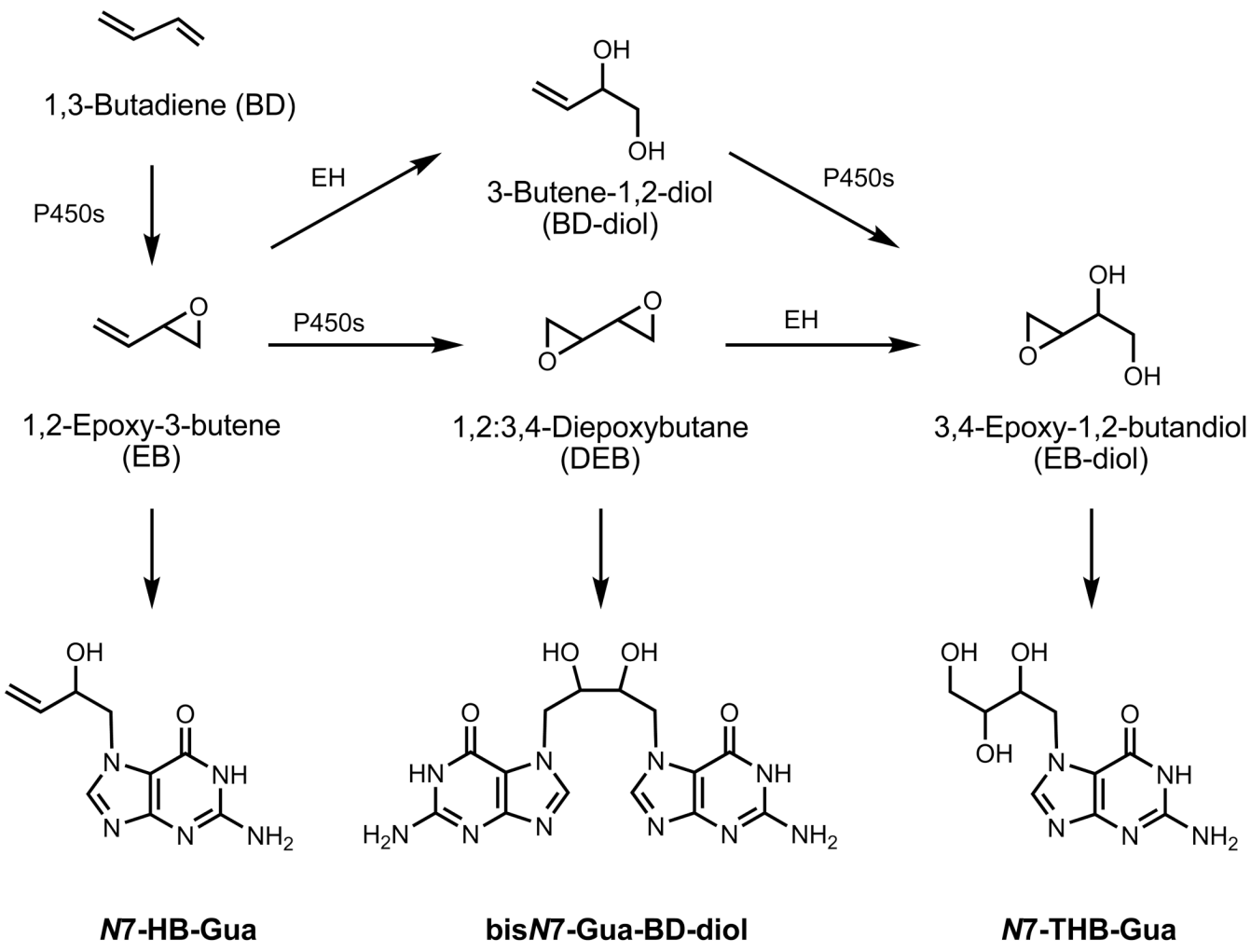

Figure 3.

Example of olefin metabolism and formation of *N*7-guanine adducts illustrated on BD. BD is metabolized by P450s to several epoxides that form DNA adducts including *N*7-guanine adducts. Shown are *N*7-HB-Gua, bis*N*7-Gua-BD-diol and THB-Gua as representative *N*7-guanine adducts formed from EB, DEB and EB-diol, respectively.

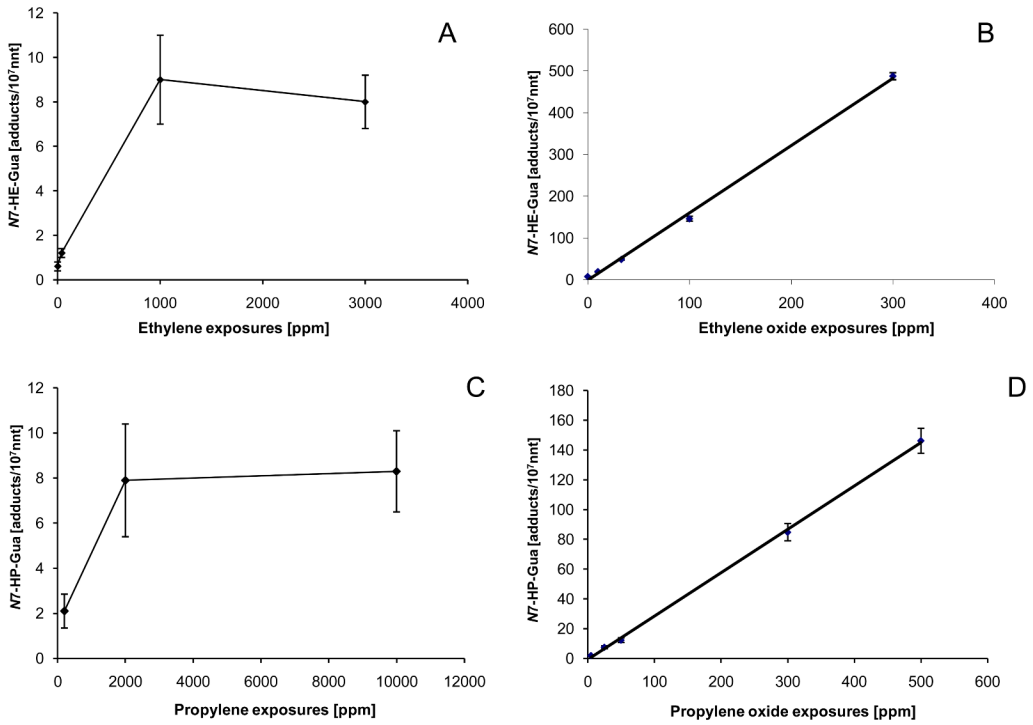

Figure 4.

Comparison of the dose-responses for formation of *N*7-hydroxyalkyl-Gua adducts following repeated exposure to the olefins ethylene (A) and propylene (C), and their epoxy metabolites EO (B) and PO (C), respectively. Formation of *N*7-guanine adducts is much more efficient for the epoxide metabolite compared to the parent olefin. Data are from rat lung after 20 days inhalation exposures to olefins and their epoxides [33,127,133,149].

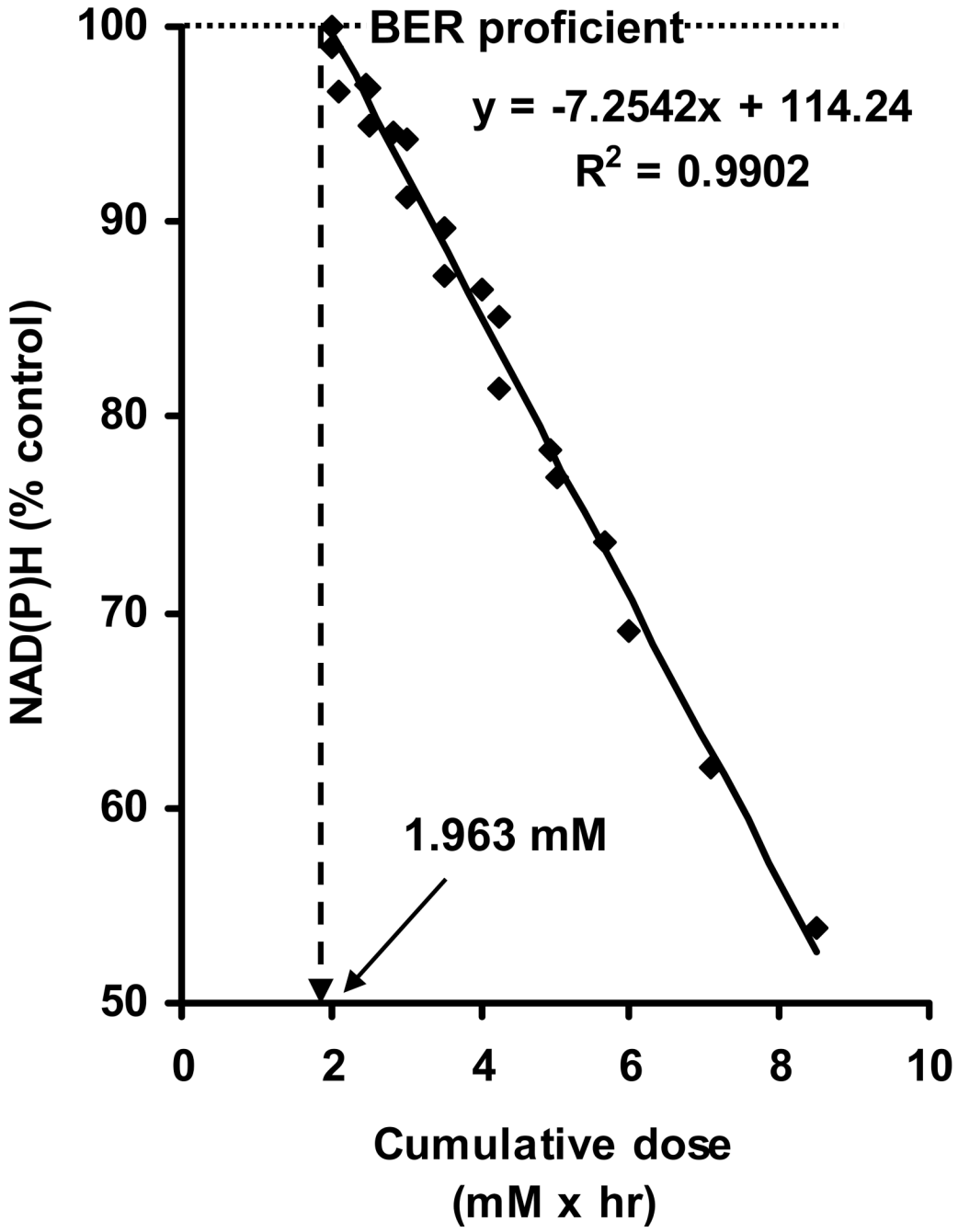

Figure 5.

Determination of the number of *N*7-Me-Gua adducts present with an imbalance in BER. NAD(P)H values for CHO AA8 cells were plotted against the corresponding cumulative dose (the product of mM MMS and exposure duration). The X value corresponding to the intersection of the resulting linear regression line and y = 100 (*i.e.*, 100% NAD(P)H relative to controls) was determined to be the start of an imbalance in BER. In the case of AA8 cells, exposure to 1.96 mM MMS for 1 hr initiated the imbalance of BER (unpublished results from Pachkowski *et al*).

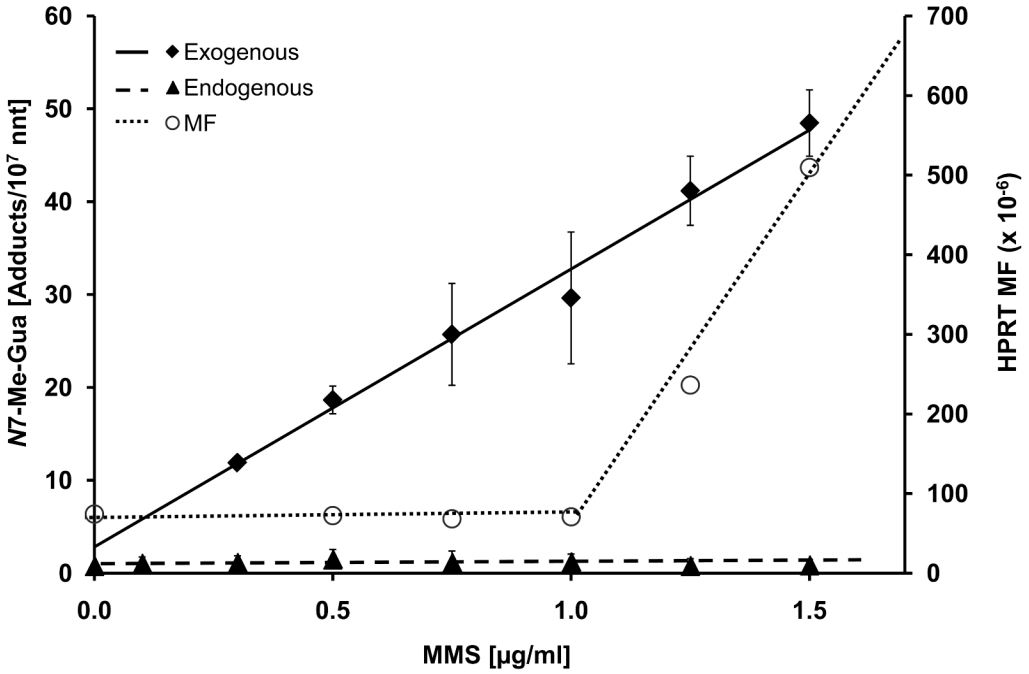

Figure 6.

Formation of endogenous and exogenous *N*7-Me-Gua in AHH-1 cells exposed to [$^{13}C_2$] MMS for 24 h (Modified from Swenberg et al [27]). Exogenous *N*7-Me-Gua adducts were distinguished form endogenous *N*7-Me-Gua base on mass differences due to stable isotope labeled [$^{13}C_2$] MMS used for cell treatment. Adduct amounts were compared to mutation frequency in HPRT gene as reported by Doak et al. [283].

Table 1

Relative reactivity [%] of nucleophilic sites in DNA

Chemical	*N*7-Gua	O^6-Gua	N^2-*Gua*	*N*3-Ade	*N*1Ade/N^6 Ade	*N*3-Cyt	N^4-Cyt	Reference
PO	100	0.5	-	4.4/10	3.5	2	-	[269,270]
EO	100	-	-	10	10	1	-	[125]
AGE	100	-	-	11	14	6	-	[271]
ECH	100				9	4		[125]
SO	100		4.5	10	16	5	2	[125]
EB	*100*			*15*	*25*	*1.5*		[153,272,273]
MNU	*100*	*10*	-	*12*	*1.2*	*0.8*	-	[274,275]
MMS	*100*	*3.7*		*12*	*2*	-	-	[274,275]
ENU	*100*	*66*		*30*	*2*	*2*	-	[274,275]
EMS	*100*	*3.4*		*7*	*3*	*0.6*		[274,275]

AGE; allyl glycidyl ether, ECH; epichlorohydrin

Table 2

Dose response of some *N*7-guanine adducts in rats exposed to representative olefins in rat liver

Compound	Dose response Max adduct (adducts / 107)	Adduct	Reference
Ethylene	Saturates at ~7 adduct/ 10^7 nnt	*N*7-HE-Gua	[33]
Ethylene oxide	Linear up to 463.8 adducts / 10^7 nnt	*N*7-HE-Gua	[129,133]
Propylene	Saturates at 6.5 adducts / 10^7 nnt	*N*7-HP-Gua	[149]
Propylene oxide	Linear up to 915 adducts / 10^7 nnt	*N*7-HP-Gua	[127]
Butadiene	Saturates at 23 adducts / 10^7 nnt	*N*7-THB-Gua	[68]

Table 3

Amounts of *N*7-guanine adducts in human DNA

Adduct/ Tissue	Group	Method	*N*7-Gua adducts/10^7 nnt (n)	Ref
***N*7-Me-Gua**				
WBC	Age<50	^{32}P-HPLC	1.0 ± 0.9 (14)	[188]
WBC	Age >80	^{32}P-HPLC	0.8 ± 0.4 (20)	[188]
WBC	NS	^{32}P-HPLC	2.5	[191]
WBC	NS	^{32}P-AEC	2.3 (6)	[190]
WBC	S	^{32}P-AEC	11.5 (11)	[190]
WBC	NS	^{32}P-AEC	3.4 (10)	[189]
WBC	S	^{32}P-AEC	6.9 (10)	[189]
Granulocytes	NS	^{32}P-AEC	2.8 (10)	[189]
Granulocytes	S	^{32}P-AEC	4.7 (10)	[189]
Lymphocytes	NS	^{32}P-AEC	13.5 (10)	[189]
Lymphocytes	S	^{32}P-AEC	23.6 (10)	[189]
Lymphocytes		^{32}P-HPLC	6.94 (5)	[142]
Lung	Lung cancer patients	^{32}P-HPLC	6.3 ± 1.9 (5)	[146]
Bronchial tissue	Lung cancer patients	^{32}P-HPLC	6.1 ± 1.5 (5)	[146]
Lymphocytes	Lung cancer patients	^{32}P-HPLC	3.3 ± 0.9 (5)	[146]
Lung		^{32}P-HPLC	39.4 (5)	[276]
Lung		^{32}P-HPLC	2.7 (10)	[142]
Bronchial tissue	NS	^{32}P-HPLC	4.7 (6)	[190]
Bronchial tissue	S	^{32}P-HPLC	17.3 (11)	[190]
Cervical cytology	NS	ISB	0.82^b (17)	[187]
Cervical cytology	S	ISB	2.5^b (22)	[187]
lymphocytes	NS	HPLC-ECD	4.5 (8)	[193]
lymphocytes	S	HPLC-ECD	3.9 (14)	[193]
Colon mucosa		ISB & ^{32}P	1.7^b (6)	[192]
Rectal mucosa		ISB & ^{32}P	0.7^b (5)	[192]
Bronchial Lavage			11.9	[277]
Bronchial Lavage	Ex-smoker	^{32}P-HPLC	9.99 ± 20.3 (10)	[195]
Bronchial Lavage	Ex-Smokers	^{32}P-HPLC	5.59 ± 15.6 (28)	[195]
Bronchial Lavage	Never smokers	^{32}P-HPLC	0.58 ± 0.5 (6)	[195]
Bladder	Tumor		0.1	[194]
			0.5	[194]
Lung		^{32}P	2.5 (80)	[278]
Lung		^{32}P-HPLC	2.74 (10)	[142]
Lung		^{32}P	2.11 (90)	[142]
N7-alkyl-Gua				
Larynx tumor	NS	^{32}P	11.3 23.2 (5)	[196]
Larynx tumor	FS	^{32}P	23.2 ± 24.1(34)	[196]
Larynx tumor	S	^{32}P	61.8 ± 84.5 (5)	[196]
Larynx non-tumor	NS	^{32}P	8.3 ± 6.0 (3)	[196]

Adduct/ Tissue	Group	Method	*N*7-Gua adducts/10^7 nnt (n)	Ref
Larynx non-tumor	FS	^{32}P	21.1 ±18.7 (25)	[196]
Larynx non-tumor	S	^{32}P	36.3 ± 20.2 (6)	[196]
***N*7-Me-Gua *N*7-HE-Gua**				
WBC	NS	^{32}P-HPLC	2.9 ± 0.9 (8)	[146]
WBC	S	^{32}P-HPLC	3.9 ± 0.8 (11)	[146]
Lung	NS	^{32}P-HPLC	4.0 [3.6, 4.4](2)	[146]
Lung	S	^{32}P-HPLC	6.5 ± 2.2 (7)	[146]
N7-Et-Gua				
Liver			0.084(25)	[279]
Lymphocytes		^{32}P-HPLC	1.0 (5)	[142]
Lung		^{32}P-HPLC	1.46 (10)	[142]
Lung		^{32}P	1.6 (75)	[278]
N7-HE-Gua				
WBC	Controls subjects	HPLC-UV	10.5 ± 4.8[a] (5)	[141]
WBC		Immuno blot	0.65	[280]
WBC	S	Immuno blot	0.11	[280]
WBC	NS	Immuno blot	0.095	[280]
lymphocytes	Control subjects	GC-HRMS	4.85± 3.1[b] (23)	[128]
Granolocytes	Hospital Workers	GC-MS	9.6 ± 5.8 (6)	[143]
Granolocytes	Non exposed			
Granolocytes	Hospital Workers	GC-MS	13.6 ± 3.9(38)	[143]
Granolocytes	<32 ppm-hr ET			
Granolocytes	Hospital Workers	GC-MS	22.7 ± 11.7 (20)	[143]
Granolocytes	>32 ppm-hr Et			
Lung	Lung cancer patients	^{32}P-HPLC	0.8 ± 0.3 (5)	[146]
Bronchial tissue	Lung cancer patients	^{32}P-HPLC	1.0 ± 0.8 (5)	[146]
Lymphocytes	Lung cancer patients	^{32}P-HPLC	0.6 ± 0.2 (5)	[146]
N7-HOEt-Gua				
brain tissue	Non treated	HPLC-ECD	1.15 ± 0.63 (6)[b]	[281]
Brain tissues distal	DTI-015 treated	HPLC-ECD	1.6 (6)[b]	[281]
Brain tissues medial	DTI-015 treated	HPLC-ECD	5.1 (6)[b]	[281]
brain tissues adjacent	DTI-015 treated	HPLC-ECD	721 (6)[b]	[281]
RAL	Non cancer	^{32}P	2.9 (0.6-11.5)	[282]
RAL	Cholangiocarcinoma	^{32}P	7.2 (1.8-48.4)	[282]
RAL	Tumor adjacent	^{32}P	8.6 (1.2-51.6)	[282]

[a] Adduct unit was converted using following calculation: 1 fmol/ μg DNA = 3.25 adducts/ 10^7 nnt

[b] Adduct unit was converted using following calculation: 1 pmol/ μmol Guanine = 1.95 adducts/ 10^7 nnt

NS; non smokers, S; smokers, ISB; Immunoslot blot, ^{32}P; ^{32}P-postlabeling, ^{32}P-AEC; ^{32}P-postlabeling anion exchange chromatography