Biomarkers in Toxicology and Risk Assessment: Informing Critical Dose–Response Relationships

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Tremendous advances have been made in the study of biomarkers related to carcinogenesis during the past 20 years. This perspective will briefly review improvements in methodology and instrumentation that have increased our abilities to measure the formation, repair, and consequences of DNA adducts. These biomarkers of exposure, along with surrogates such as protein adducts, have greatly improved our understanding of species differences in metabolism and effects of chemical stability and DNA repair on tissue differences in molecular dose. During this same time frame, improvements in assays for biomarkers of effect have provided better data and an improved understanding of the dose responses for both gene and chromosomal mutations. A framework analysis approach was used to examine the mode of action of genotoxic chemicals and the default assumption that cancer can be expected to be linear at very low doses. This analysis showed that biomarkers of exposure are usually linear at low doses, with the exception being when identical adducts are formed endogenously. Whereas biomarkers of exposure extrapolate down to zero, biomarkers of effect can only be interpolated back to the spontaneous or background number of mutations. The likely explanation for this major difference is that at high exposures, the biology that results in mutagenesis is driven by DNA damage resulting from the chemical exposure. In contrast, at very low exposures, the biology that results in mutagenesis is driven by endogenous DNA damage. The shapes of the dose−response curves for biomarkers of exposure and effect can be very different, with biomarkers of effect better informing quantitative estimates of risk for cancer, a disease that results from multiple mutations. It is also clear, however, that low dose data on mutagenesis are needed for many more chemicals.

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1. Introduction

Biomarkers have been used in medicine and toxicology for many years to assist in diagnosing and staging disease. Examples of this include measurements of blood pressure and heart rate, blood cholesterol, and liver enzymes. While these types of measurements have not traditionally been called biomarkers, they clearly fit current definitions. An NIH Study Group defined "biomarkers" as a characteristic that is objectively measured and evaluated as an indicator of normal physiologic processes, pathologic processes, or pharmacologic responses to a therapeutic intervention (*1*). Today, the term usually refers to "molecular or cellular biomarkers". This perspective will focus on a small subset of biomarkers that is being used to better understand the role of reactive molecules that produce DNA adducts thought to be involved in carcinogenesis and related biomarkers such as protein adducts and metabolites excreted in the urine. These biomarkers represent *biomarkers of exposure*. Many DNA adducts can result in mutations if DNA replication takes place before repair. Mutations, at either the gene or the chromosome level, are irreversible changes in DNA structure that alter its genetic information content. Unlike DNA adducts, mutations cannot be repaired and are heritable in the progeny of the originally mutated cell. Mutations represent *biomarkers of effect*.

Consequently, molecular and cellular biomarkers are also being incorporated into the assessment of cancer risk (*2, 3*). For example, dose–response relationships of DNA adducts and protein adducts are proposed to extend the range of observable data for risk assessment. There is general consensus that

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mutations are the primary mechanism involved in carcinogenesis (*4*); yet, the dose–response of this biomarker of effect has not received the same degree of consideration as have adducts in cancer risk assessment, even though their use could be highly informative. Dose–response relationships of mutations have primarily been addressed at high doses, utilizing in vitro prokaryotic and eukaryotic systems that have been optimized to undergo mutations. Most studies on mutational end points have been related to hazard identification. However, methods are now available to measure mutational end points in cells or whole animals (including humans), where they arise under normal physiological conditions. This perspective will review some of the basic tenets involved with the underlying science that determines the molecular dose of DNA and protein adducts, the relationships between macromolecular adducts and mutations, and the important role that mutational biomarkers should have in cancer risk assessment.

2. Methods for Measuring DNA and Protein Adducts

Methods for studying DNA adducts have greatly advanced during the past 40 years. Early studies utilized exposure to radiolabeled carcinogens and column chromatography to examine the formation of adducts. These studies usually had a throughput of one sample per day, a detection limit of 1 adduct per 10^6 nucleotides, and a cost of ~$100 per animal due to the requirement for custom synthesis of radiolabeled carcinogen (*5, 6*). This approach was followed by the development of HPLC coupled with fluorescence, radioimmunoassay, or ELISA, which increased throughput, greatly reduced costs due to the elimination of custom radioisotope synthesis, and allowed expansion to protocols that included multiple days to months of exposure. The extended exposure protocols provided information on the steady-state concentrations of 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 (*7*). However, as compared to present day technology, these methods had limited sensitivity and some of the immunoassays were plagued by cross-reactivity.

A major breakthrough in methodology occurred in 1982, when Randerath and colleagues developed ^{32}P-postlabeling methods for DNA adducts (*8*). The limit of detection for the early ^{32}P-postlabeling assays was 1 adduct per 10^8 nucleotides, but subsequent modifications, such as the combination of ^{32}P-postlabeling with HPLC or immunoaffinity, improved the sensitivity one or more orders of magnitude by permitting larger amounts of DNA to be evaluated. ^{32}P-Postlabeling works very well for bulky adducts but is less well-suited for small or depurinating adducts. The major problems associated with this method include the lack of specific chemical identity and the lack of internal standards for quantitation. The number of PubMed citations found using "^{32}P-postlabeling" and "DNA adducts" as the search strategy shows that the ^{32}P-postlabeling method gained widespread use during the mid-90s, with up to 76 citations per year. This number has now declined to ~20 citations per year.

The methods that are currently in highest use for detecting and quantifying biomarkers employ mass spectrometry. Earlier studies utilized GC-MS, but now, the vast majority of research quantifying DNA adducts is done with LC-MS/MS. The application of mass spectrometry for DNA adducts has been recently reviewed by Singh and Farmer (*9*) and by Koc and Swenberg (*10*). 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 nucleotides. A major advantage of GC- and LC-MS/MS methods is the use of chemical-specific stable isotope internal standards for quantitation. The greatest sensitivity for measuring DNA adducts is achieved with accelerator mass spectrometry, which can quantitate down to 1 adduct per 10^{12} nucleosides. While this method is extremely sensitive, it requires the use of ^{14}C-labeled chemical and has limited access due to the expense of the equipment (*11*). Citations in PubMed for "mass spectrometry" and "DNA adducts" are now averaging ~50 per year.

Mass spectrometry has been the major method used for quantitating protein adducts for many years. This primarily stems from Törnqvist's application of Edman degradation to the study of N-terminal valine adducts of globin using GC-MS/MS (*12*). Major chemicals that have been studied with this approach include ethylene oxide (EO), propylene oxide, butadiene, and acrylamide. More recently, similar approaches have been modified to use LC-MS/MS (*13, 14*). Albumin adducts represent another common biomarker of proteins, using the cleavage of cysteine adducts (*15*).

3. DNA Adducts are Biomarkers of Exposure

The availability of highly sensitive analytical methods has provided tremendous insight into the formation, persistence, and repair of DNA adducts induced by a large number of chemicals. By understanding the molecular dose of such adducts in different cells and tissues, major inroads can be achieved to understand the metabolism and mode of action (MOA)[1] of individual chemicals across species (*16*). In addition to DNA adducts, similar information can be gained from studies of protein adducts and chemical metabolites in urine and plasma. Examples of such data sets are discussed below.

Extensive research has been conducted on the molecular dosimetry of aflatoxin in rats and humans, with measurements of DNA adducts, protein adducts, and urinary excretion of both adducts and metabolites. The earlier literature was reviewed by Busby and Wogan (*17*). Aflatoxin B1 (AFB1) forms adducts at the N-7 position of guanine. These adducts can depurinate and be excreted in the urine, but they also form the ring-opened FAPY adduct, which is persistent and mutagenic (*18*). Knowledge gained from studies on aflatoxin led Groopman and colleagues to hypothesize that it would be possible to reduce the molecular dose of DNA adducts by inducing detoxication of the AFB1 epoxide with chemicals that induce glutathione pathways (*19*). They demonstrated that a 62% decrease in DNA adducts resulted in >99% reduction in preneoplastic foci and the elimination of hepatic carcinomas and adenomas (*19*). More recent studies have shown that urinary excretion of AFB1-N-7 guanine adducts can be greatly reduced in humans with dietary exposure to aflatoxin by administering oltipraz or broccoli sprouts (*20*). It is believed that the incidence of liver cancer

[1]Abbreviations: EO, ethylene oxide; AFB1, aflatoxin B1; VC, vinyl chloride; HRMS, high-resolution mass spectrometry; MGMT, methylguanine methyl transferase; DPX, DNA–protein cross-links; ROS, reactive oxygen species; 8-OH-dG, 7,8-dihydro-8-oxo-2′-deoxyguanosine; M_1G, pyrimido[1,2-*a*]purin-10(3*H*)one; ϵdA, 1,N^6-ethenodeoxyadenosine; ϵG, N^2,3-ethenoguanine; MOA, mode of action; FISH, fluorescent in situ hybridization; MN, micronuclei; PCR, polymerase chain reaction; *Hprt*, hypoxanthine-guanine phosphoribosyltransferase; *Tk*, thymidine kinase; GPA, glycophorin-A; TCR, T-cell receptor; U.S. EPA, United States Envrironmental Protection Agency; PBPK, physiologically based pharmacokinetic; N7-GA-Gua, N-7-(2-carbamoyl-2-hydroxyethyl)guanine; MMS, methylmethanesulphonate; N7-MeG, N7-methylguanine; N7-HEG, N7-2-hydroxyethylguanine; HEVal, hydroxyethyl valine; RFLP, restriction fragment length polymorphism.

will be reduced and the latency of this disease will be increased from 40 years of age to a cancer of much older individuals through the use of such strategies.

Vinyl chloride (VC) DNA adducts have been investigated in depth using ^{32}P-postlabeling and mass spectrometry (*21–24*). An unexpected finding was that identical DNA adducts were present in tissues of control animals and humans with no known exposure to VC (*25, 26*). These identical adducts were shown to result from lipid peroxidation (*27, 28*). Molecular dosimetry studies in rats demonstrated supralinear dose responses associated with saturation of metabolic activation of VC (*26*). Because of the exquisite sensitivity of the immunoaffinity GC-high-resolution mass spectrometry (HRMS) approach used, it was possible to interpolate expected relationships for endogenous adducts and those that would be associated with current day occupational exposure levels of VC. These researchers also utilized exposures to [$^{13}C_2$]VC, so that endogenous and exogenous adducts could be measured in the same animals (*29*). This unique approach demonstrated that large numbers of exogenous [$^{13}C_2$]-N^2,3-ethenoguanine were formed in the liver but not in the brain. They also confirmed the relationships between endogenous and exogenous adducts.

Numerous studies have been conducted on the DNA adducts of olefins and their epoxides, including EO, propylene oxide, and butadiene (*30–37*). The major adducts induced by these chemicals occur at the N-7 position of guanine. As discussed earlier, steady-state concentrations of N-7 guanine adducts are achieved following 7–10 days of exposure. When exposures were to the epoxides, linear responses were found (*30, 31, 34, 35*). In contrast, when exposures were to the olefins, supralinear responses were present (*32, 36, 38, 39*). EO is of particular interest, because all animals, including humans, form this genotoxic chemical endogenously. Its molecular dosimetry has been studied in globin and DNA, as well as in exhaled breath. Steady-state concentrations of globin adducts in humans have been measured (*40*), while N-7-(2-hydroxyethyl)guanine has been measured in rats, mice, and humans (*33, 38, 41–43*).

In the case of butadiene, marked species differences were noted, with mice producing greater numbers of DNA adducts than rats (*36*). Whereas N-1 deoxyadenosine adducts of butadiene and their rearrangement to N^6 deoxyadenosine and deamination to N-1 deoxyinosine adducts had been investigated in vitro and shown to be highly mutagenic, these adducts have been below detection limits in tissues from highly exposed mice, suggesting that they are either not formed or are rapidly repaired. More recently, studies on butadiene have been expanded to include globin adducts of the diepoxide (*44, 45*). Mice form much higher amounts of the diepoxide globin adducts than rats. Even lower numbers of diepoxide globin adducts are formed in humans. No evidence for gender differences was present in mice, rats, or humans. New LC-MS/MS methods have been used to demonstrate the formation of DNA−DNA cross-links of butadiene diepoxide in mice (*46*) and DNA–protein cross-links (DPX) between butadiene diepoxide and methylguanine methyl transferase (MGMT) protein in vitro (*47*).

The formation, persistence, and repair of DNA−DNA cross-links and DPX are two areas of molecular dosimetry that have been greatly understudied. While several DNA−DNA cross-links have been characterized, there is limited information on the quantitation of these important lesions. Both types of adducts form from exogenous and endogenous electrophiles, including environmental chemicals, cancer chemotherapeutic agents, and oxidative stress. DPX have been investigated with methods that rely on physical chemistry, use of detergents, and Comet assays,

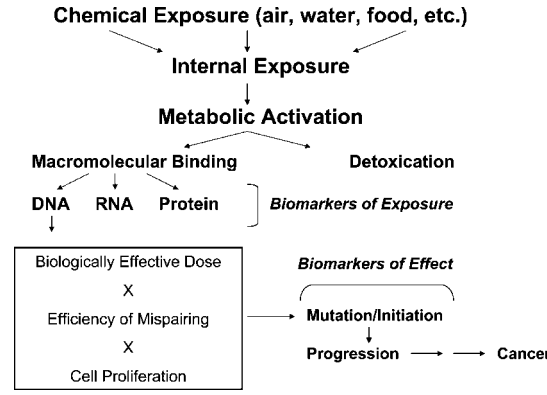

Figure 1. Scheme depicting the steps involved in the molecular dose of biomarkers of exposure and effect.

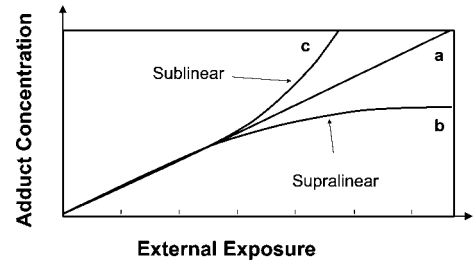

Figure 2. Graph showing lines representing (a) a linear dose response, (b) a supralinear dose response exhibited by saturation of metabolic activation, and (c) a sublinear dose response indicating saturation of detoxication.

but little is known regarding the specific formation or repair of these lesions. Part of the problem lies in the fact that many different proteins are likely to be involved, making target selection difficult. In vitro studies with isolated proteins have demonstrated specific DPX that can be formed, but there is little or no quantitative data on such effects in cells or tissues. Likewise, little is known about the repair of DNA−DNA cross-links or DPX. For example, is the protein cleaved to an amino acid or to a short peptide? If so, do physical chemistry and detergent methods differentiate these from normal DNA? Can individual amino acid−nucleoside cross-links be examined as a quantitative approach?

As will be discussed later, studies on molecular dosimetry provide critical information that is relevant to conducting mechanistically based risk assessments. Many of these examples have established dose–response relationships for DNA and protein adducts following exposure protocols similar to those used in animal carcinogenicity bioassays. The molecular dose integrates such important processes as absorption, distribution, metabolism, detoxication, and DNA repair, as generalized in Figure 1. Such dose responses include linear, sublinear, and supralinear shapes as illustrated in Figure 2. In general, sublinear responses are found at doses that exhibit impaired detoxication or DNA repair, resulting in a disproportionately increased number of adducts per unit dose at high doses. In contrast, supralinear responses are associated with saturation of metabolic activation, where fewer electrophiles are formed per unit dose at high exposures. At doses below either of these nonlinear processes, however, DNA adducts are expected to be linear functions of administered doses.

Furthermore, the site of formation of the electrophilic metabolite, combined with its chemical stability, influences the cellular and tissue distribution of DNA adducts. For example, the "methyl carbonium" ion of dimethyl nitrosamine and chloroethylene oxide arising from the metabolism of VC are

highly unstable electrophiles and do not persist long enough to form adducts in tissues distant to the site of metabolic activation. In contrast, the epoxides formed from the metabolic activation of ethylene, propylene, butadiene, acrylamide, and acrylonitrile are relatively stable, circulate in the blood, and form similar amounts of DNA adducts in many tissues and protein adducts in albumin and globin.

DNA and protein adducts also vary greatly in their half-lives. In the case of DNA adducts, the half-life depends on the chemical stability of the adduct under study, DNA repair, and cell death. Examples of unstable DNA adducts include the N-7 adducts of guanine and the N-3 adducts of adenine arising from simple alkylating agents (*48*) and the N-7 adenine adducts of estrogen quinones (*49*). Tissue-specific differences in DNA repair can greatly affect the molecular dose, as exemplified by the early experiments on O^6-alkylguanine in liver vs brain (*5, 6, 50*), where hepatocytes actively repair these adducts, but the brain has little repair. Thus, following single exposures, the molecular dose of adducts decreases rapidly in repair-proficient tissues but remains relatively stable in repair-deficient tissues.

When exposures mimic subacute to chronic exposure, DNA adducts will increase over time until a steady-state concentration is attained, where the number of adducts formed each day equal the number that are lost or repaired. For highly unstable or rapidly repaired DNA adducts such as N-3 methyladenine or N-7 estrogen adducts on adenine, steady-state concentrations may be achieved as quickly as following the first daily dose. In the case of N-7 alkylguanine adducts, steady-state concentrations are achieved in 7–10 days (*51–54*). More persistent adducts, such as O^4-ethyl thymidine, accumulate over a period of 4 weeks (*55*), while O^6-methylguanine in the brain continued to accumulate over 6 weeks of dosing (*50*).

Protein adducts are not subject to repair but also vary in half-life, depending upon the chemical stability of the adducts and the lifespan of the protein. The two most studied proteins are albumin and globin. Albumin has a half-life of 12–21 days in different species, while globin has a half-life of 42 days in mice, 63 days in rats, and 120 days in humans. As scientists begin examining the adduction of new proteins that may be involved in signal transduction, protein lifespan and targeted destruction through pathways such as ubiquitination will become increasingly important parameters to understand.

In summary, the interplay between electrophile formation and stability, adduct stability and repair, protein lifespan, and cell death determine the distribution, shape, and time course of the molecular dose being measured.

4. Oxidative DNA Damage

Oxidative stress is a common state in pathophysiology, where the number of reactive oxygen species (ROS) being formed exceeds those being detoxified. It is thought to play an important role in many diseases including cancer, neurodegeneration, and aging (*56*). Many endogenous processes, as well as exposure to both endogenous and environmental chemicals or their metabolites, are known to induce ROS through redox cycling. ROS interact with many cellular constituents, including lipids and DNA. In addition, ROS are involved in signaling pathways associated with cell death and cell proliferation (*57*). DNA damage induced by ROS and cell proliferation is thought to be two of the primary MOAs for carcinogenesis by nongenotoxic environmental chemicals, but they may also be important MOAs for genotoxic chemicals. Nongenotoxic carcinogens present major problems for risk assessment, since the shapes of their dose–response curves at low exposures are not yet deducible from mechanistic principles.

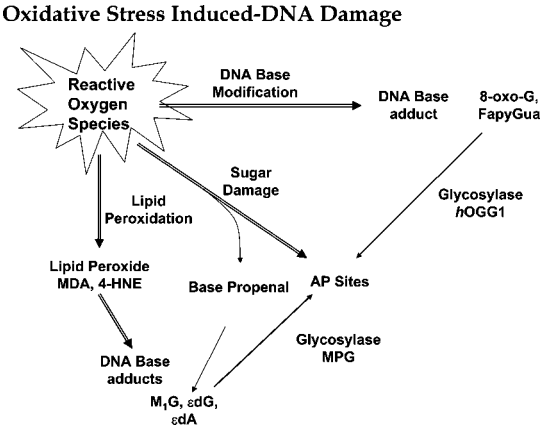

Figure 3. Scheme depicting the various steps and adducts in oxidative stress-induced DNA damage.

Endogenous DNA adducts that result from ROS are always present in genomic DNA (Figure 3). Thus, some risk of mutation is always present. This nonzero background causes uncertainty when regulatory agencies such as the United States Environmental Protection Agency (U.S. EPA) are required to extrapolate risks for hazardous chemicals that are thought to produce ROS as an important MOA for toxicity and/or carcinogenicity. The implications of background mutations for risk assessment will be discussed in relationship to low numbers of DNA adducts later in this perspective.

The DNA damage caused by ROS has been implicated in a myriad of diseases, cancer, and aging (*58–60*). Various oxidative DNA lesions have been characterized and investigated for their role in the pathogenesis of adverse health effects and disease. One of the most studied biomarkers for oxidative DNA damage is 7,8-dihydro-8-oxo-2′-deoxyguanosine (8-OH-dG). It is formed in relatively high amounts, and sensitive LC-MS/MS methods are now available for its analysis. However, the measurement of a single lesion such as 8-OH-dG as a biomarker of oxidative DNA damage might be misleading because it may not reflect the overall spectrum of DNA damage and the potential for mutations. In addition, the accumulation of different oxidative DNA lesions could be affected by DNA repair, which is mediated by different pathways depending on the type of DNA lesion. Therefore, it is desirable to have a panel of biomarkers specific for different types of ROS-induced damage to monitor oxidative DNA damage when investigating the role of ROS in the pathogenesis of disease. While base oxidation and abasic sites are the most frequent insults to DNA, substantial evidence supports that secondary DNA damage produced from byproducts of primary DNA damage or lipid membrane damage may play an important role in mutation (*26, 61*). Base propenal, a byproduct of deoxyribose oxidation, has been identified as a key intermediate, producing the exocyclic DNA adduct, pyrimido[1 2-*a*]purin-10(3*H*)one (M_1G) (*62*). More frequently, ROS can abstract a hydrogen from polyunsaturated fatty acids to produce a carbon-centered fatty acid radical, which can be further oxidized to form a lipid peroxy radical. Lipid peroxy radicals abstract hydrogen from neighboring phospholipids, producing additional lipid peroxy radicals (*63, 64*). As a result, there may be up to 2 orders of magnitude amplification in the production of free radicals and many reactive byproducts such as malondialdehyde, 4-hydroxy-nonenal, crotonaldehyde, and acrolein, which can damage genomic DNA (*65*). Lipid peroxidation-induced DNA adducts include exocyclic purine adducts

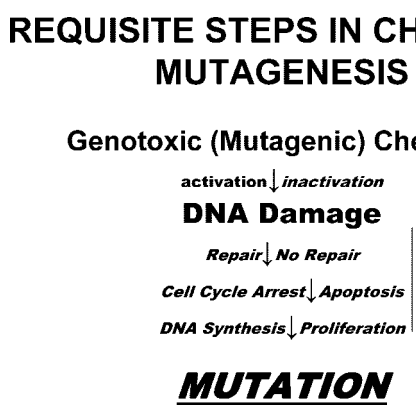

Figure 4. Requisite steps in chemical mutagenesis.

such as 1,N^6-ethenodeoxyadenosine (ϵdA), N^2,3-ethenoguanine (ϵG), and M_1G (*66*). Therefore, genomic DNA in the living cell is under constant assault by numerous endogenous mutagens that can damage DNA bases or the sugar–phosphate backbone. To counteract deleterious effects of oxidative DNA lesions, cells are equipped with a complicated network of DNA repair pathways (*67*). However, little is known about the comparative rates and efficiency of repair for this wide spectrum of oxidative DNA lesions. Differences in formation and repair will result in different molecular doses and will affect the induction and types of mutations and chromosome damage that result in toxicity and carcinogenesis.

5. Mutations as Biomarkers of Effect

The process of DNA replication is characterized by high fidelity, as orderly cell growth requires that the genetic information, encoded in DNA, be precisely transmitted from cell generation to cell generation. However, mistakes do occur, altering the primary structure of this critical molecule. Such alterations are mutations, which permanently change the genetic information of the cell and, assuming it is nonlethal, in all progeny cells.

Mutations, although rare, are always occurring. When arising without identifiable cause, they are termed "spontaneous", although "background" is a better term. Background mutations may be the result of DNA replication errors (*68*) or DNA damage (adducts) arising from endogenously produced chemicals. Processes that chemically alter DNA or produce potentially mutagenic chemicals include oxidation, methylation, deamination, and depurination, as well as normal metabolism that produces additional electrophilic compounds, for example, EO (*58, 69–73*). Superimposed on this background are the mutations resulting from exogenous agents, which may be physical (e.g., ionizing radiation), biological (e.g., viruses), or chemical. These different agents, or products that they produce, interact with the DNA, producing the adducts described above, which are the substrates for the exogenous mutations. It is important to note, however, that the DNA adducts themselves are not the mutations. It is the cells that process DNA adducts, either repairing them or not. If a cell is unsuccessful in repairing DNA damage, it may continue on without effect, die, or mutate. Figure 4 shows the importance of cell functions in processing DNA adducts to mutations.

Mutations encompass all irreversible changes in DNA primary structure and may be manifest at the gene or chromosome level. The former are submicroscopic changes, while the latter are usually observable with a light microscope. Although, by convention, the term mutation has been applied to gene level changes and the term chromosome aberration applied to chromosomal level events, the extent of an alteration in DNA structure may be a continuum, with these two different manifestations merging into each other. Mutations, in this nomenclature, usually refer to single gene effects, while chromosome aberrations may affect expression of several genes.

Distinguishing between gene and chromosome level DNA changes, or at least the extent of the DNA change involved in a mutation, does have some utility for predicting the shapes of the dose−response curves for inducing such effects. For example, single gene mutations resulting from a base substitution or a small deletion or insertion (point mutations) can arise from the mishandling of an unrepaired DNA adduct (of the appropriate kind) during DNA replication. This can be during scheduled, or S phase, replication, or during repair replication. A "targeted" mutation is then induced at the site of DNA damage (adduct), without requiring the cooperative effects of several sites of DNA damage to produce the mutation (*74*). It is important to remember, however, that the DNA adduct must remain in the DNA during its replication (i.e., be unrepaired) and that the cell must mishandle the adduct (e.g., cause a mispairing) for a mutation to occur. Exogenously induced point mutations may well show linearity at low doses, although this does not imply that mutation responses go down linearly through zero. The slope of the dose–response curve for mutations, the point on this curve where the induced mutation frequency exceeds the spontaneous background and, indeed, even the likelihood that a mutation will occur, depends on the mutagenic potency of the DNA adduct and the cell's capacity for handling the damage. The capacity of a single DNA adduct to induce a targeted point mutation, without requiring cooperation from additional adducts, has been misinterpreted to indicate that one chemical hit can result in cancer. This ignores the ultimate source of the mutation, that is, the cell and its processing of DNA adducts, either handling or mishandling them, and, furthermore, equates point mutations with cancer. Ignoring either leads to distortions.

Although point mutations may not require the cooperative effects of more than one DNA adduct, large gene deletions involving kilobase lengths of DNA often do, requiring at least one chemical hit that is processed by the cell into a double-stranded DNA break for each of the two ends of the deletion, with subsequent rejoining of the ends. This processing occurs during DNA replication. The dose–response curve here may best be described as linear-quadratic, with the linear portion being due to background or pre-existing DNA adducts. For these same reasons, chromosome aberrations that involve actual translocations between two different chromosomes or segments of chromosomes, or interstitial deletions, are likely to be two chemical hit events and show linear-quadratic dose responses (*75*). Simple chromatid breaks, as seen by the light microscope, can be single chemical hit events, but most are also lethal. Aneuploidy, which is the gain or loss of one or more whole chromosomes, can well result from damage to spindle proteins that control chromosome segregation and, because of the multiplicity of targets, can show a threshold response (*76*). As should be obvious, there is no necessary relationship between the shapes of the dose–response curves for DNA adducts and those for the resultant mutations, should any be produced.

Mutations are the critical biomarkers for cancer risk assessment for agents having a mutagenic MOA because, in this case, mutation is the critical key event. When mutations are observed, the shapes of the dose–response curves provide some aids for extrapolating to potential cancer effects, recognizing that there are many events required beyond the mutations for the evolution

of cancer. Mutational dose–response curves that are quadratic at higher or intermediate doses allow more accurate determinations of points of departure for estimating mutagenic effects at levels below limits of detection. An important corollary to a mutagenic MOA for cancer is that a chemical that induces cancer by this MOA cannot induce cancer under conditions where it does not induce mutations.

6. Methods for Monitoring Mutations

Cytogenetic studies have long been used to assess chromosome level mutations in animals and humans (*77, 78*). The methods have evolved from simple analyses of stained chromosomes, to studies of banded chromosomes, to investigations involving fluorescent in situ hybridization (FISH) to identify specific portions of chromosomes involved in chromosome translocations. Techniques that detect either whole or parts of chromosomes present in the cytoplasm of cells (micronuclei = MN) allow more rapid detection of some classes of chromosome aberrations (*78*). These events are detected in either bone marrow or peripheral blood cells (immature red cells or lymphocytes) of animals but only in peripheral blood lymphocytes in humans. Their implications and interpretations differ somewhat, depending on the target cell type. More recently, polymerase chain reaction (PCR) methods have been used to identify specific chromosome translocations in the blood of humans (*79*). To the extent that the latter becomes possible, it may allow detection of cancer (leukemia)-specific chromosome level mutations to be monitored in humans, whereas the other cytogenetic methods detect only nonspecific reporter events. However, even for the nonspecific changes, a remarkable statistical association has been shown between the frequencies of chromosome aberrations and the subsequent occurrences of cancer (*80*).

Somatic mutations at the gene level can be detected in both cancer-specific genes and in reporter genes in animals and humans. Potentially informative target mutations for monitoring, therefore, include those in oncogenes, for example, *ras*, and those in tumor suppressor genes, for example, P53, depending on the species (*81, 82*). Cancer-specific mutations can be detected in normal, noncancer cells or in actual tumors. Studies of cancer mutations in tumors that include characterizations of the kinds of mutations present (molecular mutational spectra) have been used to infer causation (*83, 84*). Measuring mutations in cancer genes in normal individuals (animals or humans) has the advantage of relevance, in that events directly on the pathway to cancer are being monitored. However, there are two potential disadvantages to using such genes for this purpose. First, there may be ambiguity in that it is not always clear if the mutations are truly arising in noncancer cells or if they are indicators of early malignancy. This can have psychological consequences when monitoring healthy worker populations. The second potential disadvantage is that cancer-specific mutations may change cell growth characteristics, allowing cells carrying the mutation to overgrow normal cells and change the relationship between mutants and mutations.

In contrast to mutations in cancer-specific genes, those that arise in reporter genes, which also serve to quantify and characterize in vivo mutations, have no role in disease processes. In animals, both endogenous and transgenes can serve as reporters. The former include the hypoxanthine-guanine phosphoribosyltransferase (*Hprt*) and, more recently, the thymidine kinase (*Tk*) genes (*85, 86*). *Hprt* is located on the X-chromosome, while *Tk* is autosomal. These locations have some implications as to the kinds of mutations that can be detected

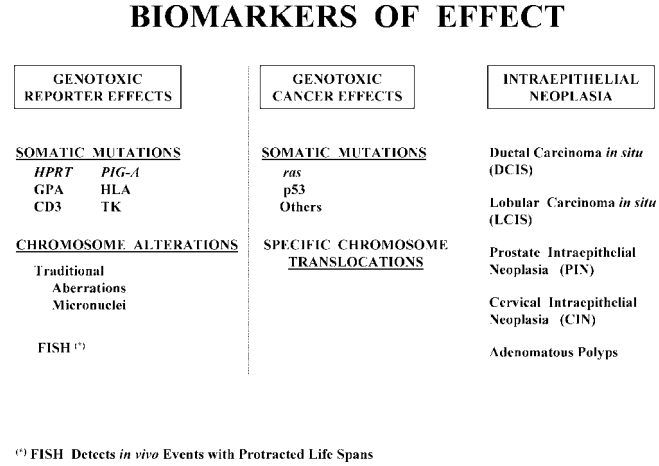

Figure 5. Representative biomarkers of effect in humans.

in that there can be no homologous crossing-over events for X-chromsomal genes. Both *Hprt* and *Tk* mutations are assayed in blood cells. By contrast, there are now transgenic rodent models available for the analysis of somatic gene mutations. The *lacI* and *lacZ* bacterial genes have both been introduced into rodents, allowing them to serve as reporters of mutations in the animals (*87*). An advantage to using these reporter genes as biomarkers of mutation is that mutations can be detected in almost any tissue, allowing correlations to be made between target tissues for cancer and for mutations. Unfortunately, there are also several disadvantages to using these transgenes for mutational studies. First, they are quite small in size and cannot record large deletion mutations. This important class is therefore missed. Furthermore, there appear to be quite high background mutation rates leading to relatively high background mutation frequencies. This lowers the sensitivities for detecting induced mutations resulting from exogenous chemicals. Lastly, these bacterial genes are normally not transcribed; therefore, damage in them is not repaired by transcription-coupled DNA repair systems (*88*).

There are currently four reporter gene/cell systems that have been used to some degree for human in vivo mutation studies (*81, 89*). These include the glycophorin-A (GPA) gene, studied in red blood cells, the *HPRT* gene, the *HLA* genes, and the T-cell receptor (TCR) genes, all measured in T-lymphocytes. The GPA red cell system employs cytometry to enumerate variant cells but does not allow molecular characterization of the mutations. All of the T-cell systems potentially allow for mutant isolation and molecular studies to define mutational spectra. Practically, most data on somatic gene mutations in humans have come from studies of *HPRT* mutations. Figure 5 illustrates the different reporter and cancer genes available for human mutagenicity monitoring and puts them into the context of the progression of mutations to early malignancy.

7. The Use of Biomarkers to Inform Risk Assessment

Biomarkers have long been used in risk assessment, public health, and clinical medicine. An early example was the general knowledge that high cholesterol increased the probability of heart disease and stroke. As mentioned in the introduction, the U.S. EPA Guidelines for Carcinogen Risk Assessment (*2*) propose that DNA and protein adducts represent biomarkers of internal dose that extend knowledge on the shape of the dose response below what can be achieved from cancer bioassays. Indeed, there are now numerous studies that demonstrate DNA and/or globin adducts over exposures covering several orders

of magnitude in experimental animals. Likewise, many studies on globin adducts in humans have examined exposures ranging from high occupational exposures to low environmental, dietary, and endogenous sources of exposure to EO and acrylamide (*90, 91*). ^{32}P-postlabeling and accelerator mass spectrometry have been used to examine the molecular dose of tamoxifen in laboratory rats, mice, and monkeys, as well as in tissues of patients receiving it as a cancer chemotherapeutic (*11, 92*).

The U.S. EPA Guidelines for Carcinogen Risk Assessment (*2*) state that the assessment of risk should be based on the best scientific knowledge available for a given chemical and favor the use of a biologically based assessment of risk when possible. The guidelines also utilize the "Framework Analysis of Mode of Action" to identify the MOA for individual chemicals in a transparent manner to examine the key events that are thought to drive the carcinogenic response(s). The "Framework Analysis of Mode of Action" was first developed in a meeting of government and academic scientists in 1997 and published by Sonich-Mullin in 2002 (*93*). It has subsequently been expanded with case studies and considerations of potential human relevance (*94, 95*). Most examples to date have been for specific chemicals, and some have utilized molecular dosimetry data. Such framework analyses state the proposed MOA, identify the key events that are thought to drive the development of cancer, compare the dose response of these key events with that of carcinogenesis, assess the consistency between studies, draw conclusions on the weight of evidence for the proposed MOA, and identify gaps and uncertainties that remain. Most recently, a draft "Framework Analysis of Genotoxicity" has been prepared and submitted for peer review by the U.S. EPA (*3*). This document addresses the key events involved and how to apply weight of evidence analyses but does not go into dose−response relationships. The rationale for not examining this critical issue was not discussed in the draft document but will be examined below.

The same approach and principles can readily be applied to examine default assumptions that are used in risk assessment. Major default assumptions include (i) that chemicals that are carcinogenic to animals will be carcinogenic to humans, (ii) that humans are as sensitive as the most sensitive animal species, and (iii) that genotoxic chemicals will have a risk that is linear from high to very low exposures.

8. Framework Analysis of Low Dose Linearity of Mutations

In the section below, the "framework analysis" approach will be used to examine the default that identifying a chemical as "genotoxic" supports a linear assessment of cancer risk (Figure 6). Each of the "key events" in the framework analysis for low-dose linearity of mutations will be examined for its contribution to identifying information important for risk assessment, from both the standpoint of species similarities and differences in metabolism and the key events that are representative of the heritable alterations that drive the carcinogenic response.

8.1. Key Event #1: Genotoxicity. A chemical is considered genotoxic/mutagenic if the weight of evidence indicates that it can cause heritable mutations, chromosome aberrations, or MN using an approach similar to that proposed by the U.S. EPA (*3*). Data used in the weight of evidence decision are usually generated in short-term tests in bacteria such as the Ames test, in vitro assays such as *Hprt*, or *Tk* mutation assays, micronucleus formation, and chromosome alterations. These studies are usually conducted early in the time line for toxicology studies on a given chemical. They usually represent qualitative information relevant to hazard identification, rather than quantitative data useful in addressing the shape of the dose−response relationship for risk assessment. Hazard identification determines if a chemical can cause a genetic change under any condition. There may also be in vivo data on similar gene or chromosome mutational end points in animals and occasionally in humans. When such data are available, they should be used to inform the dose response as will be discussed below under Key Event #3.

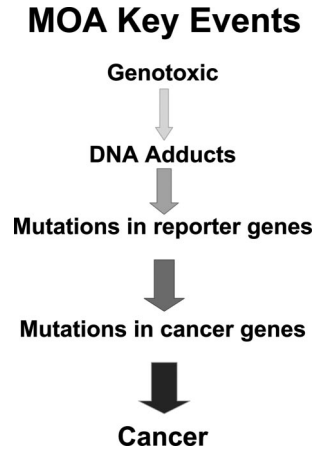

Figure 6. Key events in assessing the mutagenic MOA.

8.2. Key Event #2: DNA Adducts. DNA adducts provide important information on the molecular dose and its tissue distribution. As discussed earlier, such data can be highly informative about the metabolism of the chemical and the repair of adducts. Molecular dosimetry can also be used to examine species similarities and differences in adduct formation and repair, as well as inflections in the dose–response curve. While nonlinearities of either the supra- or the sublinear form can and do occur at high doses, when exposures go below doses that saturate activation, detoxication, or repair, DNA adducts are expected to be linear. It is important to recognize that DNA adducts are biomarkers of exposure, not heritable mutations. These biomarkers of exposure are likely to support the default of linear risk at low doses. Exceptions to the expected linearity of DNA adducts arise when chemically induced adducts are identical to adducts that arise from endogenous sources such as ROS. At higher exposures, adducts arising from the exogenous exposures will dominate the molecular dose. In contrast, at low exposures, the likelihood that a mutation will arise from the exogenous adducts becomes de minimus as compared to the large molecular dose formed from normal cellular metabolism. Two examples of endogenous chemicals include the etheno adducts of VC that are also formed by ROS and the hydroxyethyl adducts of EO that arise from ethylene production from gut microflora, lipid peroxidation, and endogenous metabolism. In such cases, the molecular dose from exogenous exposures will effectively plateau at the concentration present in unexposed individuals, and the biologic effects of de minimus exposures below endogenous amounts will be lost in the noise of the background. For example, recent studies from Brown and colleagues showed that rat endogenous N7-HEG adducts ranged from 1.1 to 3.5 adducts per 10^8 nucleosides (*32*). Administration of single doses of [^{14}C]EO ranging from 0.0001 up to 0.01 mg/kg and measurement of N7-HEG by accelerator mass spectrometry were roughly linear but were de minimus relative to the endogenous levels of N7-HEG (Brown et al., personal communication).

8.3. Key Event #3: Mutations in Reporter Genes. In contrast to DNA adducts, which are normally linear down to

zero, mutation frequencies in genes such as *Hprt* or *Tk*, chromosome aberrations, or MN have dose responses for heritable mutations that terminate at a nonzero spontaneous background rate. These dose responses for heritable mutations at the gene or chromosomal level may be linear or nonlinear but do not go to zero in unexposed cells. As mentioned earlier, the cells in animals, humans, and cell culture are subjected to continuous endogenous DNA damage arising from oxidative stress, depurination, and other endogenous electrophiles, such as EO. At "high" exposures to genotoxic chemicals or metabolites, the DNA damage induced by the exogenous exposure drives the biology of mutagenesis at the gene and chromosomal level. However, when the exposures are low, the biology driving the induction of mutations is derived from the endogenous sources. The primary issue for risk assessment revolves around the definition of "low", that is, what constitutes a de minimus level of exogenous exposure that would lead to a de minimus increment in mutations and increased risk? This has been a topic of discussion for many years (*96*). The BEIR Report III (*97*) concluded that one should "use simple linear interpolation between the lowest reliable dose data and the spontaneous...rate." Experimental studies on mutagenesis and carcinogenesis are usually confined to exposures covering 1−2 orders of magnitude, and these doses are frequently high to establish hazard identification. In contrast, risk assessment extrapolations frequently cover up to 6 orders of magnitude. Recent data on the molecular dose of DNA and protein adducts cover 4–5 orders of magnitude and show linear responses. Better data on the dose response for mutations and the subsequent utilization of these data in quantitative risk assessment will provide critical scientific information on dose−response relationships that are highly relevant for carcinogenesis. These data will be superior to DNA and protein adducts, since they represent biomarkers of effect, rather than biomarkers of exposure.

The dose–response curve for mutagenic end points may not be linear even if the associated DNA adduct response is linear. Indeed, mutagenic compounds may very well possess dose–response thresholds, that is, positive doses below which there is no increase in mutation frequency above that observed in unexposed control animals. To illustrate this, the data for three chemicals with different dose responses will be examined in more detail.

Acrylamide has been studied by toxicologists for many years. The primary area of investigation focused on its ability to induce neurotoxicity (*98, 99*). Acrylamide was evaluated for carcinogenicity in two traditional rat bioassays and found to cause several tumor types at oral doses ranging from 0.5 to 3.0 mg/kg/day (*100, 101*). More recently, its epoxide metabolite, glycidamide, was also shown to form globin adducts and DNA adducts. In 2001, Törnqvist and colleagues were conducting studies on tunnel construction workers to investigate acrylamide exposures and neurotoxicity (*102, 103*). The workers were exposed to significant amounts of acrylamide as a result of its use in waterproofing the construction area. For comparison with the workers, globin adducts of controls with no known exposure to acrylamide were examined and found to have readily detectable amounts of acrylamide and glycidamide globin adducts in their blood (*104*). Many additional researchers began studies to determine the sources of exposure. Ultimately, it was shown that when carbohydrates are exposed to high temperatures, the Maillard reaction converts asparagine and sugars to acrylamide (*105*). Thus, humans are constantly exposed to low amounts of acrylamide in the daily diet.

Several research groups have gone on to develop major data sets for metabolism in animals and humans, molecular dosimetry of DNA adducts in experimental animals, gene and chromosome mutagenicity data, and physiologically based pharmacokinetic (PBPK) models for cross-species extrapolation. The major DNA adduct formed by acrylamide, N-7-(2-carbamoyl-2-hydroxyethyl)guanine (N7-GA-Gua) adduct, is formed by glycidamide, the epoxide of acrylamide that results from P450 metabolism. N7-GA-Gua is not a promutagenic DNA adduct but can chemically depurinate, leaving an apurinic site. Studies by Doerge and colleagues have shown that it reaches steady-state concentrations in 7–14 days, similar to many related N7-Gua adducts. The major route of loss of this adduct is chemical depurination. Steady-state concentrations following dietary exposure to 1 mg/kg/day are 90–100 per 10^8 nucleotides (*53, 54*). The same group of investigators demonstrated that autoclaved laboratory chow contained amounts of acrylamide that were 7-fold higher than irradiated chow (*106*). This provided one of the rare examples of very low exposure. Mice consuming irradiated chow had a daily dose of 1.4 μg/kg/day, which resulted in steady-state concentrations of 1 N7-GA-Gua adducts per 10^8 nucleotides, while the 1 mg/kg/day mice had 90–100 N7-GA-Gua adducts per 10^8 nucleotides adducts and 2.6 mg/kg resulted in 240 adducts/10^8 nucleotides (*53, 107*). Thus, a 700- and 1820-fold higher dose gave a 100- and 240-fold greater number of adducts. Single exposure studies by Doerge and coworkers demonstrated that doses ranging from 1 to 100 mg/kg were also linear (*108*). Thus, there are data on the molecular dose of N7-GA-Gua that show a linear response over more than 4 orders of magnitude.

The ability of acrylamide to induce a variety of gene and chromosomal mutations also has been evaluated. Doses that induced positive mutation studies following 3–4 weeks of drinking water exposure were conducted between 20 and 100 mg/kg/day (*109*). Micronucleus, *hprt*, and *cII* mutations in Big Blue mice were significantly increased at the high dose, while only *hprt* was increased at the 19–25 mg/kg dose. The only study that has examined lower doses of acrylamide to date was that of Abramsson-Zetterberg (*108*). This investigation provided data for single exposures of 0, 1, 3, 6, 12, 24, and 30 mg/kg. The authors concluded that acrylamide caused a linear increase in MN. However, examination of the data clearly shows that only doses of 6 mg/kg and higher resulted in a significant increase in MN. Specifically, 1 or 3 mg/kg was not different from the control mice, even though they received a dose of acrylamide that was ∼700- and 2100-fold higher than the controls. Figure 7 compares the findings of Doerge and colleagues to Abramsson-Zetterberg. It demonstrates that the number of N7-GA-Gua adducts, a biomarker of exposure, increases more than 200-fold, while the number of MN does not change (*53, 108*).

Methylmethanesulphonate (MMS) is a well-studied mutagen that has recently been evaluated for low dose effects in the *Hprt* and *Tk* genes (*110*). In addition, we have examined the molecular dose of the major DNA adduct, N7-methylguanine (N7-MeG), over the same dose range studied by Doak et al. (*110*) using [$^{13}C^2H_3$]MMS. Figure 8 compares the data for mutations at *HPRT* and DNA adducts in AHH-1 cells. In addition, the endogenous N7-MeG was quantitated at each dose (9.8 ± 4.6 N7-MeG/10^8 ntds). It is readily apparent that the shape of the mutation curves and the shape of the molecular dose of N7-MeG are different. The DNA adducts are linear over the entire dose response and intersect with the identical

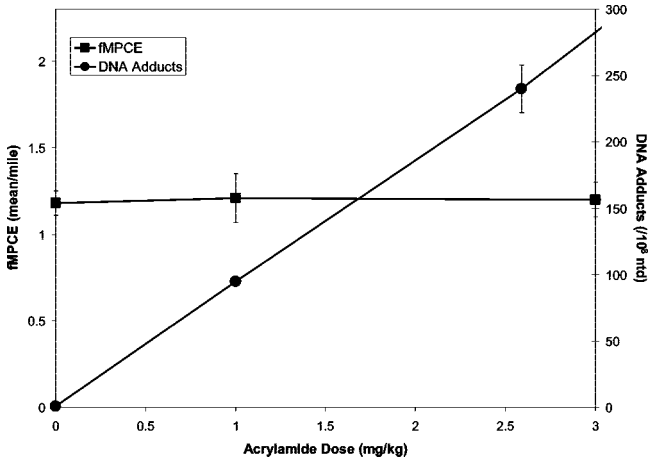

Figure 7. Relationships between DNA adducts and micronucleus induction in mice exposed to carcinogenic doses of acrylamide. Polychromatic erythrocytes in peripheral blood (fMPCE) (■) from Abramsson-Zetterberg (*108*). DNA adducts (●) are 0.0014 μg/kg from Twaddle et al. (*106*), 1 mg/kg from Tareke et al. (*107*), and 2.6 mg/kg from Young et al. (*54*).

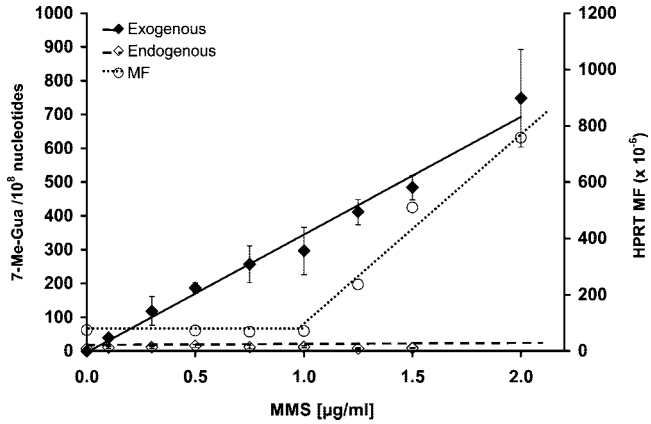

Figure 8. Comparison of N7-methyl guanine DNA adducts and HPRT mutations in AHH-1 cells exposed to MMS for 24 h. The endogenous adducts are N-7Me-G (◊), while the exogenous adducts are [$^{13}C^2H_3$]-7Me-G (♦). The Hprt mutant frequency is shown as ○ (*110*).

endogenous adduct, while *HPRT* and *Tk* mutations, as well as micronucleus inductions, are highly sublinear (*110*).

EO is a chemical with both exogenous and endogenous exposure. The exogenous exposures come from occupational, tobacco smoke, and environmental sources, while the endogenous exposure comes from metabolism of ethylene produced by gut microflora, ROS, and metabolism. Molecular dosimetry of DNA adducts has been examined with HPLC and fluorescence detection, GC-HRMS, and LC-MS/MS. As with acrylamide and MMS, the major DNA adduct occurs at the N-7 position of guanine. Steady-state concentrations of N7-2-hydroxyethylguanine (N7-HEG) occur between 7 and 10 days of exposure. DNA adducts have also been used as molecular dosimeters for ethylene exposure, which allowed extrapolation of EO mutations to even lower exposures by utilizing the metabolism of ethylene to EO. Exposure-related increases in N7-HEG and hydroxyethyl valine (HEVal) globin adducts have been measured by several research groups and found to increase linearly with exposures to EO above endogenous concentrations. A variety of mutational end points have also been measured. Using a 4 week inhalation exposure protocol, mutations in *Hprt* were increased following EO exposures at 50 ppm and higher but not at ethylene exposures resulting in 4.5 ± 2.0, 9.0 ± 1.9, or 10.0 ± 3.0 ppm EO (Figure 9). Similar data were reported

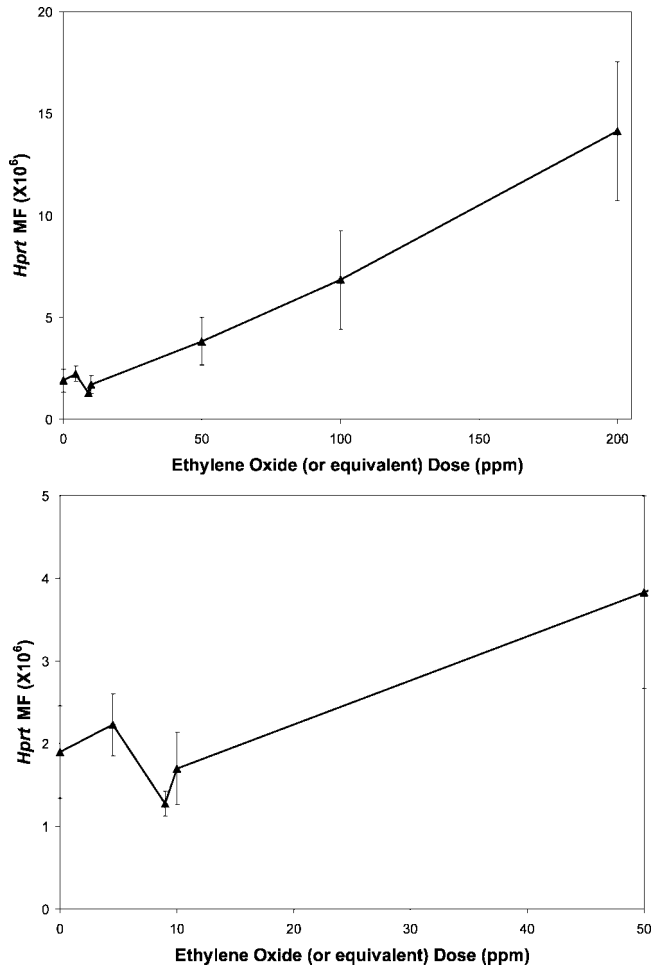

Figure 9. Comparison of Hprt mutant frequencies in mice exposed to ethylene (40, 1000, and 3000 ppm; equivalent EO exposures were calculated to be 4.5 ± 2.0, 9.0 ± 1.9, and 10.0 ± 3.0 ppm) or to EO (50, 100, and 200 ppm). Animals were exposed for 4 weeks, 6 h/day, 5 days/week. The control point is the mean ± SD of ethylene and EO controls.

by Tates et al. (*111*). Nivard et al. (*112*) have also reported important data from Drosophila comparing the relationship between N7-HEG and mutations. The wild-type flies showed that mutations reached background frequencies, while DNA adducts were linear. If nucleotide repair-deficient flies were used, the mutations also came down to background frequencies, but the exposure response was shifted to the left, demonstrating the importance of DNA repair. The system used by Nivard et al. (*112*) has an extremely high rate of cell proliferation, leaving minimal time for DNA repair. Comparisons of cell proliferation in Drosophila germ cells vs the human equivalent demonstrate that the fly's fertilized ovum undergoes very rapid cell proliferation that is ~85 times faster than the human, minimizing the effect of DNA repair.

8.4. Key Event #4: Mutations in Cancer Genes. It is now well-established that cancer results from the induction of multiple mutations in genes that control signaling pathways associated with cell proliferation, cell death, metastasis, and vascularization (*4, 113*). At the present time, little quantitative data are available on chemical exposures and these end points, particularly in noncancerous tissues. Early studies by Cerutti and colleagues utilized restriction fragment length polymorphism (RFLP)/PCR methods to demonstrate the ability of AFB1 to induce specific mutations at a p53 hot spot in HepG2 cells that had also been identified in human hepatocellular carcinomas (*114*). Mutations at other sites in p53 were also induced in

HepG2 cells by the exposure but were not related to hot spots in human tumors or to functional changes in the gene. They later showed that such increases were present in nontumorous regions of the liver in humans from geographic regions that correlated with AFB1 exposure (*115*), but the data were not considered quantitative. Furthermore, the predominant mutations in patients' tumors were different from those in nonmalignant tissue. The authors concluded that the point mutations most likely represented premalignant changes that contributed to multistage carcinogenesis.

Tumors in humans and those that were induced by many carcinogens in animals have been evaluated for such mutations. These involve point mutations in *ras*, p53, p^{16INK4}, and Rb, loss of heterozygocity in p53, etc., demonstrating that chemical carcinogenesis in test animals and humans arises via the same pathways. The pathways associated with gene and chromosome mutations in cancer genes drive the biology that results in carcinogenesis. Because the factors that result in mutations of cancer genes are not different than the factors that result in reporter gene mutations, Key Events 3 and 4 should be the most important data sets for informing the dose response for cancer risk assessment.

Unfortunately, this has not been the case as yet. One may ask, why not? There are many parts to the answer of that question. First, at one time, simplistic theories that one hit could lead to cancer were proposed (*116*). Second, limited numbers of mutagenesis studies have carried out "low" dose exposures. Third, many members of the risk assessment community fall back on oversimplified mathematical modeling that only considers the numbers of tumors in carcinogenicity bioassays, rather than integrating the complexity of the scientific data.

9. Conclusions

The development of highly specific and sensitive assays for biomarkers of exposure and effect has provided a vastly improved understanding of events related to chemical exposure, metabolism, DNA damage and repair, as well as the resultant effects on mutations. This understanding provides critical stochastic knowledge on the quantitative biologic processes that determine how chemicals cause cancer. The 2005 Cancer Risk Assessment Guidelines (*2*) clearly state that a biologically driven model is preferred for cancer risk assessment. For as long as *Chemical Research in Toxicology* has been published, we have used biomarkers of exposure to understand species, tissue, and cell type differences in DNA damage and repair. It seemed reasonable to use such biomarkers of exposure to also inform risk assessment, since it integrated exposure, metabolic activation, and detoxication. However, by using the framework analysis to carry this information forward, along with data on biomarkers of effect that measure exposure responses of gene and chromosomal mutations, it became clear that major differences exist in the shapes of the dose−response relationships for the biomarkers of exposure and biomarkers of effect. This new understanding has critical implications for cancer risk assessment, since it is well-accepted that mutations at the gene and chromosome level are major determinants in the genesis of cancer in laboratory animals and humans. While one cannot exclude a linear component for such mutational events, it is clear that they do not occur following a dose response that parallels biomarkers of exposure. On further reflection, this should not come as a surprise. It has been known for many years that some DNA adducts are either not or are only weakly promutagenic and do not cause mispairing during DNA replication. We have utilized three examples to evaluate these differences, all of which have relatively weak strength as mutagens. The literature certainly contains numerous examples of highly promutagenic DNA adducts. A similar evaluation of biomarkers of exposure vs effect will need to be conducted to determine if and how they differ in these responses. One thing is clear, however. The dose response for mutations will come into the background frequency of the controls at some point. When this happens, the biology of mutagenesis and subsequently carcinogenesis is being driven by the endogenous DNA damage that arises from ROS, endogenous EO, spontaneous depurination, and errors of DNA polymerase. Additional research will be needed to more fully understand each carcinogen, from the standpoint of molecular dose, and the exposure response for mutations. Application of a "framework analysis" approach to this issue has increased the transparency of such deliberations and identified data gaps that will further reduce the uncertainties associated with risk assessment through the use of science, rather than defaults.

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References

(1) Biomarkers Definitions Working Group (2001) Biomarkers and surrogate endpoints: Preferred definitions and conceptual framework. *Clin. Pharmacol. Ther. 69*, 89−95.

(2) U.S. Environmental Protection Agency (2005) *Guidelines for Carcinogen Risk Assessment*, U.S. Environmental Protection Agency, Washington, DC.

(3) Schoeny, R. S., Chu, M. M., Cimino, M. C., Dearfield, K. L., Kligerman, A., Keshava, N., McCarroll, N., Owen, R. D., Moore, M. M., Putzrath, R. M., and Hoffmann, E. L. (2007) *DRAFT Framework for Determining a Mutagenic Mode of Action for Carcinogenicity: Using EPA's 2005 Cancer Guidelines and Supplemental Guidance for Assessing Susceptibility from Early-Life Exposure to Carcinogens*, U.S. Environmental Protection Agency, Washington, DC.

(4) Hanahan, D., and Weinberg, R. A. (2000) The hallmarks of cancer. *Cell 100*, 57–70.

(5) Goth, R., and Rajewsky, M. F. (1974) Persistence of O^6-ethylguanine in rat-brain DNA: Correlation with nervous system-specific carcinogenesis by ethylnitrosourea. *Proc. Natl. Acad. Sci. U.S.A. 71*, 639–643.

(6) Kleihues, P., and Margison, G. P. (1974) Carcinogenicity of *N*-methyl-*N*-nitrosourea: Possible role of excision repair of O^6-methylguanine from DNA. *J. Natl. Cancer Inst. 53*, 1839–1841.

(7) Swenberg, J. A., Dyroff, M. C., Bedell, M. A., Popp, J. A., Huh, N., Kirstein, U., and Rajewsky, M. F. (1984) O^4-Ethyldeoxythymidine, but not O^6-ethyldeoxyguanosine, accumulate in hepatocyte DNA of rats exposed continuously to diethylnitrosamine. *Proc. Natl. Acad. Sci. U.S.A. 81*, 1692–1695.

(8) Gupta, R. C., Reddy, M. V., and Randerath, K. (1982) ^{32}P-postlabeling analysis of non-radioactive aromatic carcinogen-DNA adducts. *Carcinogenesis 3*, 1081–1092.

(9) Singh, R., and Farmer, P. B. (2006) Liquid chromatography-electrospray ionization mass spectrometry: The future of DNA adduct detection. *Carcinogenesis 27*, 178–196.

(10) Koc, H., and Swenberg, J. A. (2002) Applications of mass spectrometry for quantitation of DNA adducts. *J. Chromatogr. B 778*, 323–343.

(11) Brown, K., Tompkins, E. M., Boocock, D. J., Martin, E. A., Farmer, P. B., Turteltaub, K. W., Ubick, E., Hemingway, D., Horner-Glister, E., and White, I. N. (2007) Tamoxifen forms DNA adducts in human colon after administration of a single [^{14}C]-labeled therapeutic dose. *Cancer Res. 67*, 6995–7002.

(12) Törnqvist, M., Mowrer, J., Jensen, S., and Ehrenberg, L. (1986) Monitoring of environmental cancer initiators through hemoglobin adducts by a modified Edman degradation method. *Anal. Biochem. 154*, 255–266.

(13) Fennell, T. R., Sumner, S. C. J., Snyder, R. W., Burgess, J., Spicer, R., Bridson, W. E., and Friedman, M. A. (2005) Metabolism and hemoglobin adduct formation of acrylamide in humans. *Toxicol. Sci. 85*, 447–459.

(14) Fennell, T. R., Snyder, R. W., Krol, W. L., and Sumner, S. C. (2003) Comparison of the hemoglobin adducts formed by administration of *N*-methylolacrylamide and acrylamide to rats. *Toxicol. Sci. 71*, 164–175.

(15) Rappaport, S. M., Waidyanatha, S., Qu, Q., Shore, R., Jin, X., Cohen, B., Chen, L. C., Melikian, A. A., Li, G., Yin, S., Yan, H., Xu, B., Mu, R., Li, Y., Zhang, X., and Li, K. (2002) Albumin adducts of benzene oxide and 1,4-benzoquinone as measures of human benzene metabolism. *Cancer Res. 62*, 1330–1337.

(16) La, D. K., and Swenberg, J. A. (1997) Carcinogenic alkylating agents. In *Comprehensive Toxicology, Vol. 12, Chemical Carcinogens and Anticarcinogens* (Sipes, I. G., McQueen, C. A., and Gandolfi, A. J., Eds.) pp 111–140, Elsevier Science, Oxford.

(17) Busby, W. F., and Wogan, G. N. (1984) Aflatoxins. In *Chemical Carcinogenesis* (Searle, C. E., Ed.) pp 945–1136, American Chemical Society, Washington.

(18) Smela, M. E., Hamm, M. L., Henderson, P. T., Harris, C. M., Harris, T. M., and Essigmann, J. M. (2002) The aflatoxin B(1) formamidopyrimidine adduct plays a major role in causing the types of mutations observed in human hepatocellular carcinoma. *Proc. Natl. Acad. Sci. U.S.A. 99*, 6655–6660.

(19) Roebuck, B. D., Liu, Y. L., Rogers, A. E., Groopman, J. D., and Kensler, T. W. (1991) Protection against aflatoxin B1-induced hepatocarcinogenesis in F344 rats by 5-(2-pyrazinyl)-4-methyl-1,2-dithiole-3-thione (oltipraz): Predictive role for short-term molecular dosimetry. *Cancer Res. 51*, 5501–5506.

(20) Camoirano, A., Bagnasco, M., Bennicelli, C., Cartiglia, C., Wang, J. B., Zhang, B. C., Zhu, Y. R., Qian, G. S., Egner, P. A., Jacobson, L. P., Kensler, T. W., and De Flora, S. (2001) Oltipraz chemoprevention trial in Qidong, People's Republic of China: Results of urine genotoxicity assays as related to smoking habits. *Cancer Epidemiol. Biomarkers Prev. 10*, 775–783.

(21) Barbin, A. (1999) Role of etheno DNA adducts in carcinogenesis induced by vinyl chloride in rats. In *Exocyclic DNA Adducts in Mutagenesis and Carcinogensis* (Singer, B., and Bartsch, H., Eds.) pp 303–313, IARC Scientific Publications, Lyon, France.

(22) Fedtke, N., Boucheron, J. A., Turner, M. J., and Swenberg, J. A. (1990) Vinyl chloride-induced DNA adducts. I: Quantitative determination of N^2,3-ethenoguanine based on electrophore labeling. *Carcinogenesis 11*, 1279–1285.

(23) Morinello, E. J., and Swenberg, J. A. (2000) Molecular dosimetry and repair studies of N^2,3-ethenoguanine in hepatocytes and nonparenchymal cells from rats exposed to vinyl chloride. *Toxicol. Sci. 54*, 220.

(24) Swenberg, J. A., Bogdanffy, M. S., Ham, A. J., Holt, S., Kim, A., Morinello, E. J., Ranasinghe, A., Scheller, N., and Upton, P. B. (1999) Formation and repair of DNA adducts in vinyl chloride- and vinyl fluoride-induced carcinogenesis. In *Exocyclic DNA Adducts in Mutagenesis and Carcinogenesis* (Singer, B., and Bartsch, H., Eds.) pp 29–43, IARC Scientific Publications, Lyon, France.

(25) Morinello, E. J., Ranasinghe, A., and Swenberg, J. A. (2002) Differential induction of N^2,3-ethenoguanine in rat brain and liver after exposure to vinyl chloride. *Cancer Res. 62*, 5183–5188.

(26) Morinello, E. J., Ham, A. J. L., Ranasinghe, A., Nakamura, J., Upton, P. B., and Swenberg, J. A. (2002) Molecular dosimetry and repair of N^2,3-ethenoguanine in rats exposed to vinyl chloride. *Cancer Res. 62*, 5189–5195.

(27) el Ghissassi, F., Barbin, A., Nair, J., and Bartsch, H. (1995) Formation of 1,N^6-ethenoadenine and 3,N^4-ethenocytosine by lipid peroxidation products nucleic acid bases. *Chem. Res. Toxicol. 8*, 278–283.

(28) Ham, A. J., Ranasinghe, A., Koc, H., and Swenberg, J. A. (2000) 4-Hydroxy-2-nonenal and ethyl linoleate form N^2,3-ethenoguanine under peroxidizing conditions. *Chem. Res. Toxicol. 13*, 1243–1250.

(29) Morinello, E. J., Ham, A. J. L., and Swenberg, J. A. (1999) Molecular dosimetry of N^2,3-ethenoguanine in control and vinyl chloride-exposed rats. *Toxicol. Sci. 48*, 232.

(30) Walker, V. E., Fennell, T. R., Upton, P. B., MacNeela, J. P., and Swenberg, J. A. (1993) Molecular dosimetry of DNA and hemoglobin adducts in mice and rats exposed to ethylene oxide. *Environ. Health Perspect. 99*, 11–17.

(31) Walker, V. E., Fennell, T. R., Upton, P. B., Skopek, T. R., Prevost, V., Shuker, D. E. G., and Swenberg, J. A. (1992) Molecular dosimetry of ethylene oxide: Formation and persistence of 7-(2-hydroxyethyl)guanine in DNA following tepeated exposures of rats and mice. *Cancer Res. 52*, 4328–4334.

(32) Rusyn, I., Asakura, S., Li, Y., Kosyk, O., Koc, H., Nakamura, J., Upton, P. B., and Swenberg, J. A. (2005) Effects of ethylene oxide and ethylene inhalation on DNA adducts, apurinic/apyrimidinic sites and expression of base excision DNA repair genes in rat brain, spleen, and liver. *DNA Repair 4*, 1099–1110.

(33) Marsden, D. A., Jones, D. J. L., Lamb, J. H., Tompkins, E. M., Farmer, P. B., and Brown, K. (2007) Determination of endogenous and exogenously derived N^7-(2-hydroxyethyl)guanine adducts in ethylene oxide-treated rats. *Chem. Res. Toxicol. 20*, 290–299.

(34) Ríos-Blanco, M. N., Faller, T. H., Nakamura, J., Kessler, W., Kreuzer, P. E., Ranasinghe, A., Filser, J. G., and Swenberg, J. A. (2000) Quantitation of DNA and hemoglobin adducts and apurinic/apyrimidinic sites in tissues of F344 rats exposed to propylene oxide by inhalation. *Carcinogenesis 21*, 2011–2018.

(35) Ríos-Blanco, M. N., Ranasinghe, A., Lee, M. S., Faller, T., Filser, J. G., and Swenberg, J. A. (2003) Molecular dosimetry of N^7-(2-hydroxypropyl)guanine in tissues of F344 rats after inhalation exposure to propylene oxide. *Carcinogenesis 24*, 1233–1238.

(36) Koc, H., Tretyakova, N. Y., Walker, V. E., Henderson, R. F., and Swenberg, J. A. (1999) Molecular dosimetry of N-7 guanine adduct formation in mice and rats exposed to 1,3-butadiene. *Chem. Res. Toxicol. 12*, 566–574.

(37) Koivisto, P., Kilpelainen, I., Rasanen, I., Adler, I. D., Pacchierotti, F., and Peltonen, K. (1999) Butadiene diolepoxide- and diepoxybutane-derived DNA adducts at N7-guanine: A high occurrence of diolepoxide-derived adducts in mouse lung after 1,3-butadiene exposure. *Carcinogenesis 20*, 1253–1259.

(38) Wu, K. Y., Ranasinghe, A., Upton, P. B., Walker, V. E., and Swenberg, J. A. (1999) Molecular dosimetry of endogenous and ethylene oxide-induced N^7-(2-hydroxyethyl) guanine formation in tissues of rodents. *Carcinogenesis 20*, 1787–1792.

(39) Pottenger, L. H., Malley, L. A., Bogdanffy, M. S., Donner, E. M., Upton, P. B., Li, Y., Walker, V. E., Harkema, J. R., Banton, M. I., and Swenberg, J. A. (2007) Evaluation of effects from repeated inhalation exposure of F344 rats to high concentrations of propylene. *Toxicol. Sci. 97*, 336–347.

(40) Boogaard, P. J., Rocchi, P. S., and Van Sittert, N. J. (1999) Biomonitoring of exposure to ethylene oxide and propylene oxide by determination of hemoglobin adducts: Correlations between airborne exposure and adduct levels. *Int. Arch. Occup. Environ. Health 72*, 142–150.

(41) Wu, K. Y., Scheller, N., Ranasinghe, A., Yen, T. Y., Sangaiah, R., Giese, R., and Swenberg, J. A. (1999) A gas chromatography/electron capture/negative chemical ionization high-resolution mass spectrometry method for analysis of endogenous and exogenous N^7-(2-hydroxyethyl)guanine in rodents and its potential for human biological monitoring. *Chem. Res. Toxicol. 12*, 722–729.

(42) Bolt, H. M. (1996) Quantification of endogenous carcinogens—The ethylene oxide paradox. *Biochem. Pharmacol. 52*, 1–5.

(43) Bolt, H. M., Leutbecher, M., and Golka, K. (1997) A note on the physiological background of the ethylene oxide adduct 7-(2-hydroxyethyl)guanine in DNA from human blood. *Arch. Toxicol. 71*, 719–721.

(44) Boysen, G., Georgieva, N. I., Upton, P. B., Jayaraj, K., Li, Y., Walker, V. E., and Swenberg, J. A. (2004) Analysis of diepoxide-specific cyclic *N*-terminal globin adducts in mice and rats after inhalation exposure to 1,3-butadiene. *Cancer Res. 64*, 8517–8520.

(45) Boysen, G., Georgieva, N. I., Upton, P. B., Walker, V. E., and Swenberg, J. A. (2007) N-terminal globin adducts as biomarkers for formation of butadiene derived epoxides. *Chem.-Biol. Interact. 166*, 84–92.

(46) Goggin, M., Loeber, R., Park, S., Walker, V., Wickliffe, J., and Tretyakova, N. (2007) HPLC-ESI+-MS/MS analysis of N^7-guanine-N^7-guanine DNA cross-links in tissues of mice exposed to 1,3-butadiene. *Chem. Res. Toxicol. 20*, 839–847.

(47) Loeber, R., Rajesh, M., Fang, Q., Pegg, A. E., and Tretyakova, N. (2006) Cross-linking of the human DNA repair protein O^6-alkylguanine DNA alkyltransferase to DNA in the presence of 1,2,3,4-diepoxybutane. *Chem. Res. Toxicol. 19*, 645–654.

(48) Singer, B., Grunberger, D., and Sinha, N. (1983) *Molecular Biology of Mutagens and Carcinogens*, Plenum Press, New York.

(49) Cavalieri, E. L., Stack, D. E., Devanesan, P. D., Todorovic, R., Dwivedy, I., Higginbotham, S., Johansson, S. L., Patil, K. D., Gross, M. L., Gooden, J. K., Ramanathan, R., Cerny, R. L., and Rogan, E. G. (1997) Molecular origin of cancer: Catechol estrogen-3,4-quinones as endogenous tumor initiators. *Proc. Natl. Acad. Sci. U.S.A. 94*, 10937–10942.

(50) Kleihues, P., and Bucheler, J. (1977) Long-term persistence of O^6-methylguanine in rat brain DNA. *Nature 269*, 625–626.

(51) Lewis, J. G., and Swenberg, J. A. (1980) Differential repair of O^6-methylguanine in DNA of rat hepatocytes and nonparenchymal cells. *Nature 288*, 185–187.

(52) Walker, V. E., Wu, K. Y., Upton, P. B., Ranasinghe, A., Scheller, N., Cho, M. H., Vergnes, J. S., Skopek, T. R., and Swenberg, J. A. (2000) Biomarkers of exposure and effect as indicators of potential carcinogenic risk arising from *in vivo* metabolism of ethylene to ethylene oxide. *Carcinogenesis 21*, 1661–1669.

(53) Doerge, D. R., Gamboa da Costa, G., McDaniel, L. P., Churchwell, M. I., Twaddle, N. C., and Beland, F. A. (2005) DNA adducts derived

from administration of acrylamide and glycidamide to mice and rats. *Mutat. Res. 580*, 131–141.

(54) Young, J. F., Luecke, R. H., and Doerge, D. R. (2007) Physiologically based pharmacokinetic/pharmacodynamic model for acrylamide and its metabolites in mice, rats, and humans. *Chem. Res. Toxicol. 20*, 388–399.

(55) Boucheron, J. A., Richardson, F. C., Morgan, P. H., and Swenberg, J. A. (1987) Molecular dosimetry of O^4-ethyldexoythymidine in rats continuously exposed to diethylnitrosamine. *Cancer Res. 47*, 1577–1581.

(56) Ames, B. N. (1989) Endogenous DNA damage as related to cancer and aging. *Mutat. Res. 214*, 41–46.

(57) Burdick, A. D., Davis, J. W., Liu, K. J., Hudson, L. G., Shi, H., Monske, M. L., and Burchiel, S. W. (2003) Benzo(a)pyrene quinones increase cell proliferation, generate reactive oxygen species, and transactivate the epidermal growth factor receptor in breast epithelial cells. *Cancer Res. 63*, 7825–7833.

(58) Ames, B. N. (1983) Dietary carcinogens and anticarcinogens. *Science 221*, 1256–1264.

(59) Taylor, A., and Davies, K. J. (1987) Protein oxidation and loss of protease activity may lead to cataract formation in the aged lens. *Free Radical Biol Med. 3*, 371–377.

(60) Nakamura, J., and Swenberg, J. A. (1999) Endogenous apurinic/apyrimidinic sites in genomic DNA of mammalian tissues. *Cancer Res. 59*, 2522–2526.

(61) Ham, A. J. L., Ranasinghe, A., Koc, H., and Swenberg, J. A. (2000) 4-Hydroxy-2-nonenal and ethyl linoleate form N^2,3-ethenoguanine under peroxidizing conditions. *Chem. Res. Toxicol. 13*, 1243–1250.

(62) Dedon, P. C., Plastaras, J. P., Rouzer, C. A., and Marnett, L. J. (1998) Indirect mutagenesis by oxidative DNA damage: Formation of the pyrimidopurinone adduct of deoxyguanosine by base propenal. *Proc. Natl. Acad. Sci. U.S.A. 95*, 11113–11116.

(63) Maas, R. L., Ingram, C. D., Porter, A. T., Oates, J. A., Taber, D. F., and Brash, A. R. (1985) Investigation of the chemical conversion of hydroperoxyeicosatetraenoate to leukotriene epoxide using stereospecifically labeled arachidonic acid. Comparison with the enzymatic reaction. *J. Biol. Chem. 260*, 4217–4228.

(64) Pryor, W. A., and Porter, N. A. (1990) Suggested mechanisms for the production of 4-hydroxy-2-nonenal from the autoxidation of polyunsaturated fatty acids. *Free Radical. Biol. Med. 8*, 541–543.

(65) Schauenstein, E., and Esterbauer, H. (1978) Formation and properties of reactive aldehydes. *Ciba Found. Symp.* 225–244.

(66) Marnett, L. J., and Burcham, P. C. (1993) Endogenous DNA adducts: Potential and paradox. *Chem. Res. Toxicol. 6*, 771–785.

(67) Friedberg, E. C. (2003) DNA damage and repair. *Nature 421*, 436–440.

(68) Kunkel, T. A., and Bebenek, K. (2000) DNA replication fidelity. *Annu. Rev. Biochem. 69*, 497–529.

(69) Ehrenberg, L., Osterman-Golkar, S., Segerbäck, D., Svensson, K., and Calleman, C. J. (1977) Evaluation of genetic risks of alkylating agents. III. Alkylation of haemoglobin after metabolic conversion of ethene to ethene oxide in vivo. *Mutat. Res. 45*, 175–184.

(70) Harman, D. (1981) The aging process. *Proc. Natl. Acad. Sci. U.S.A. 78*, 7124–7128.

(71) Saul, R. L., and Ames, B. N. (1986) Background levels of DNA damage in the population. In *Mechanisms of DNA Damage and Repair: Implications for Carcinogenesis and Risk Assessment* (Simic, M. G., Grossman, L., and Upton, A. C., Eds.) pp 529–535, Plenum Press, New York.

(72) Törnqvist, M., Almberg, J. G., Bergmark, E., Nilsson, S., and Osterman-Golkar, S. M. (1989) Ethylene oxide doses in ethene-exposed fruit store workers. *Scand. J. Work Environ. Health 15*, 436–438.

(73) Filser, J. G., Denk, B., Törnqvist, M., Kessler, W., and Ehrenberg, L. (1992) Pharmacokinetics of ethylene in man: Body burden with ethylene oxide and hydroxyethylation of hemoglobin due to endogenous and environmental ethylene. *Arch. Toxicol. 66*, 157–163.

(74) Heflich, R. H. (1991) Chemical mutagens. In *Genetic Toxicology* (Li, A. P., and Heflich, R. H., Eds.) CRC Press, Boca Raton.

(75) Preston, R. J., Fennell, T. R., Leber, A. P., Sielken, R. L., and Swenberg, J. A. (1995) Reconsideration of the genetic risk assessment for ethylene oxide exposures. *Environ. Mol. Mutagen. 26*, 189–202.

(76) Parry, J. M., Jenkins, G. J., Haddad, F., Bourner, R., and Parry, E. M. (2000) In vitro and in vivo extrapolations of genotoxin exposures: Consideration of factors which influence dose-response thresholds. *Mutat. Res. 464*, 53–63.

(77) Albertini, R. J., Anderson, D., Douglas, G. R., Hagmar, L., Hemminki, K., Merlo, F., Natarajan, A. T., Norppa, H., Shuker, D. E., Tice, R., Waters, M. D., and Aitio, A. (2000) IPCS guidelines for the monitoring of genotoxic effects of carcinogens in humans. International Programme on Chemical Safety. *Mutat. Res. 463*, 111–172.

(78) Tucker, J. D., Eastmond, D. A., and Littlefield, L. G. (1997) Cytogenetic end-points as biological dosimeters and predictors of risk in epidemiological studies. *IARC Sci. Publ. 142*, 185–200.

(79) Smith, M. T., Skibola, C. F., Allan, J. M., and Morgan, G. J. (2004) Causal models of leukaemia and lymphoma. *IARC Sci. Publ. 157*, 373–392.

(80) Hagmar, L., Stromberg, U., Tinnerberg, H., and Mikoczy, Z. (2004) Epidemiological evaluation of cytogenetic biomarkers as potential surrogate end-points for cancer. *IARC Sci. Publ. 157*, 207–215.

(81) Albertini, R. J., and Hayes, R. B. (1997) Somatic cell mutations in cancer epidemiology. *IARC Sci. Publ. 142*, 159–184.

(82) Parsons, B. L., Delongchamp, R. R., Beland, F. A., and Heflich, R. H. (2006) Levels of H-ras codon 61 CAA to AAA mutation: Response to 4-ABP-treatment and Pms2-deficiency. *Mutagenesis 21*, 29–34.

(83) Olivier, M., Hussain, S. P., Caron, D. F., Hainaut, P., and Harris, C. C. (2004) TP53 mutation spectra and load: a tool for generating hypotheses on the etiology of cancer. *IARC Sci. Publ. 157*, 247–270.

(84) Ton, T. V., Hong, H. H., Devereux, T. R., Melnick, R. L., Sills, R. C., and Kim, Y. (2007) Evaluation of genetic alterations in cancer-related genes in lung and brain tumors from B6C3F1 mice exposed to 1,3-butadiene or chloroprene. *Chem.-Biol. Interact. 166*, 112–120.

(85) Meng, Q., Walker, D. M., McDonald, J. D., Henderson, R. F., Carter, M. M., Cook, D. L., Jr., McCash, C. L., Torres, S. M., Bauer, M. J., Seilkop, S. K., Upton, P. B., Georgieva, N. I., Boysen, G., Swenberg, J. A., and Walker, V. E. (2007) Age-, gender-, and species-dependent mutagenicity in T cells of mice and rats exposed by inhalation to 1,3-butadiene. *Chem.-Biol. Interact. 166*, 121–131.

(86) Dobrovolsky, V. N., Casciano, D. A., and Heflich, R. H. (1999) Tk+/− mouse model for detecting in vivo mutation in an endogenous, autosomal gene. *Mutat. Res. 423*, 125–136.

(87) Dean, S. W., Brooks, T. M., Burlinson, B., Mirsalis, J., Myhr, B., Recio, L., and Thybaud, V. (1999) Transgenic mouse mutation assay systems can play an important role in regulatory mutagenicity testing in vivo for the detection of site-of-contact mutagens. *Mutagenesis 14*, 141–151.

(88) Lambert, S., and Carr, A. M. (2005) Checkpoint responses to replication fork barriers. *Biochimie 87*, 591–602.

(89) Albertini, R. J. (2004) Mechanistic insights from biomarker studies: Somatic mutations and rodent/human comparisons following exposure to a potential carcinogen. *IARC Sci. Publ. 157*, 153–177.

(90) Ehrenberg, L., Granath, F., and Törnqvist, M. (1996) Macromolecule adducts as biomarkers of exposure to environmental mutagens in human populations. *Environ. Health Perspect. 104*, 423–428.

(91) Dybing, E., Farmer, P. B., Andersen, M., Fennell, T. R., Lalljie, S. P. D., Muller, D. J. G., Olin, S., Petersen, B. J., Schlatter, J., Scholz, G., Scimeca, J. A., Slimani, N., Törnqvist, M., Tuijtelaars, S., and Verger, P. (2005) Human exposure and internal dose assessments of acrylamide in food. *Food Chem. Toxicol. 43*, 365–410.

(92) Randerath, K., Moorthy, B., Mabon, N., and Sriram, P. (1994) Tamoxifen: Evidence by ^{32}P-postlabeling and use of metabolic inhibitors for two distinct pathways leading to mouse hepatic DNA adduct formation and identification of 4-hydroxytamoxifen as a proximate metabolite. *Carcinogenesis 15*, 2087–2094.

(93) Sonich-Mullin, C., Fielder, R., Wiltse, J., Baetcke, K., Dempsey, J., Fenner-Crisp, P., Grant, D., Hartley, M., Knaap, A., Kroese, D., Mangelsdorf, I., Meek, E., Rice, J. M., and Younes, M. (2001) IPCS conceptual framework for evaluating a mode of action for chemical carcinogenesis. *Regul. Toxicol. Pharmacol. 34*, 146–152.

(94) Meek, M. E., Renwick, A., Ohanian, E., Dourson, M., Lake, B., Naumann, B. D., and Vu, V. (2002) Guidelines for application of chemical-specific adjustment factors in dose/concentration-response assessment. *Toxicology 181–182*, 115–120.

(95) Boobis, A. R., Cohen, S. M., Dellarco, V., McGregor, D., Meek, M. E., Vickers, C., Willcocks, D., and Farland, W. (2006) IPCS framework for analyzing the relevance of a cancer mode of action for humans. *Crit. Rev. Toxicol. 36*, 781–792.

(96) Ehling, U. H., Averbeck, D., Cerutti, P. A., Friedman, J., Greim, H., Kolbye, A. C., Jr., and Mendelsohn, M. L. (1983) International Commission for Protection against Environmental Mutagens and Carcinogens. *Mutat. Res. 123*, 281–341.

(97) BEIR Report (1980) *The Effects on Populations of Exposure to Low Levels of Ionizing Radiation; Biological Effects of Ionizing Radiations*, National Academy Press, Washington.

(98) Spencer, P. S., and Schaumburg, H. H. (1974) A review of acrylamide neurotoxicity. Part II. Experimental animal neurotoxicity and pathologic mechanisms. *Can. J. Neurol. Sci. 1*, 152–169.

(99) Spencer, P. S., and Schaumburg, H. H. (1974) A review of acrylamide neurotoxicity. Part I. Properties, uses and human exposure. *Can. J. Neurol. Sci. 1*, 143–150.

(100) Johnson, K. A., Gorzinski, S. J., Bodner, K. M., Campbell, R. A., Wolf, C. H., Friedman, M. A., and Mast, R. W. (1986) Chronic

toxicity and oncogenicity study on acrylamide incorporated in the drinking water of Fischer 344 rats. *Toxicol. Appl. Pharmacol. 85*, 154–168.

(101) Friedman, M. A., Dulak, L. H., and Stedham, M. A. (1995) A lifetime oncogenicity study in rats with acrylamide. *Fundam. Appl. Toxicol. 27*, 95–105.

(102) Hagmar, L., Törnqvist, M., Nordander, C., Rosen, I., Bruze, M., Kautiainen, A., Magnusson, A. L., Malmberg, B., Aprea, P., Granath, F., and Axmon, A. (2001) Health effects of occupational exposure to acrylamide using hemoglobin adducts as biomarkers of internal dose. *Scand. J. Work Environ. Health 27*, 219–226.

(103) Kjuus, H., Goffeng, L. O., Heier, M. S., Sjoholm, H., Ovrebo, S., Skaug, V., Paulsson, B., Törnqvist, M., and Brudal, S. (2004) Effects on the peripheral nervous system of tunnel workers exposed to acrylamide and *N*-methylolacrylamide. *Scand. J. Work Environ. Health 30*, 21–29.

(104) Tareke, E., Rydberg, P., Karlsson, P., Eriksson, S., and Törnqvist, M. (2000) Acrylamide: A cooking carcinogen? *Chem. Res. Toxicol. 13*, 517–522.

(105) Stadler, R. H., Blank, I., Varga, N., Robert, F., Hau, J., Guy, P. A., Robert, M. C., and Riediker, S. (2002) Acrylamide from Maillard reaction products. *Nature 419*, 449–450.

(106) Twaddle, N. C., Churchwell, M. I., McDaniel, L. P., and Doerge, D. R. (2004) Autoclave sterilization produces acrylamide in rodent diets: Implications for toxicity testing. *J. Agric. Food Chem. 52*, 4344–4349.

(107) Tareke, E., Twaddle, N. C., McDaniel, L. P., Churchwell, M. I., Young, J. F., and Doerge, D. R. (2006) Relationships between biomarkers of exposure and toxicokinetics in Fischer 344 rats and B6C3F1 mice administered single doses of acrylamide and glycidamide and multiple doses of acrylamide. *Toxicol. Appl. Pharmacol. 217*, 63–75.

(108) Abramsson-Zetterberg, L. (2003) The dose-response relationship at very low doses of acrylamide is linear in the flow cytometer-based mouse micronucleus assay. *Mutat. Res. 535*, 215–222.

(109) Manjanatha, M. G., Aidoo, A., Shelton, S. D., Bishop, M. E., McDaniel, L. P., Lyn-Cook, L. E., and Doerge, D. R. (2006) Genotoxicity of acrylamide and its metabolite glycidamide administered in drinking water to male and female Big Blue mice. *Environ. Mol. Mutagen. 47*, 6–17.

(110) Doak, S. H., Jenkins, G. J., Johnson, G. E., Quick, E., Parry, E. M., and Parry, J. M. (2007) Mechanistic influences for mutation induction curves after exposure to DNA-reactive carcinogens. *Cancer Res. 67*, 3904–3911.

(111) Tates, A. D., van Dam, F. J., Natarajan, A. T., van Teylingen, C. M., de Zwart, F. A., Zwinderman, A. H., Van Sittert, N. J., Nilsen, A., Nilsen, O. G., Zahlsen, K., Magnusson, A. L., and Törnqvist, M. (1999) Measurement of *HPRT* mutations in splenic lymphocytes and haemoglobin adducts in erythrocytes of Lewis rats exposed to ethylene oxide. *Mutat. Res. 431*, 397–415.

(112) Nivard, M. J. M., Czene, K., Segerbäck, D., and Vogel, E. W. (2003) Mutagenic activity of ethylene oxide and propylene oxide under *XPG* proficient and deficient conditions in relation to *N*-7-(2-hydroxyalkyl)guanine levels in Drosophila. *Mutat. Res. 529*, 95–107.

(113) Preston, R. J., and Williams, G. M. (2005) DNA-reactive carcinogens: mode of action and human cancer hazard. *Crit. Rev. Toxicol. 35*, 673–683.

(114) Aguilar, F., Hussain, S. P., and Cerutti, P. (1993) Aflatoxin B1 induces the transversion of G-->T in codon 249 of the p53 tumor suppressor gene in human hepatocytes. *Proc. Natl. Acad. Sci. U.S.A. 90*, 8586–8590.

(115) Aguilar, F., Harris, C. C., Sun, T., Hollstein, M., and Cerutti, P. (1994) Geographic variation of *p53 m*utational profile in nonmalignant human liver. *Science 264*, 1317–1319.

(116) Iversen, S., and Arley, N. (1950) On the mechanism of experimental carcinogenesis. *Acta Pathol. Microbiol. Scand. 27*, 773–803.

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