Review

Biological Relevance of Adduct Detection to the Chemoprevention of Cancer

Ricky A. Sharma,1 and Peter B. Farmer2
1Cancer Biomarkers and Prevention Group, Oncology Department, University of Leicester, Leicester Royal Infirmary, and 2Cancer Biomarkers and Prevention Group, Biocentre, Department of Biochemistry, University of Leicester, Leicester, United Kingdom

ABSTRACT
Adducts arise from the chemical modification of bases in DNA or amino acids in proteins by toxic chemicals. Many chemicals known to be carcinogenic in humans have been shown to form adducts or to cause oxidative damage to genomic DNA in model systems. Biomarkers of carcinogenesis reflect biological events that take place between exposure to external or endogenous carcinogens and the subsequent development of cancer. Therapeutic intervention for the purpose of cancer chemoprevention may modify these biomarkers. In this article, the potential efficacy of DNA adducts as biomarkers of carcinogenesis and chemoprevention is discussed using criteria defined for phases of biomarker development. The sensitivity of adduct detection in histologically normal tissue offers opportunities for the early detection of carcinogenesis. Extensive evidence for aflatoxin B1 adducts as biomarkers of risk and progression of hepatic carcinogenesis and for oxidative DNA adducts as biomarkers of the development of prostate carcinogenesis is reviewed together with the clinical trials measuring these adducts as biomarkers of the efficacy of chemoprevention. Favorable modification of oxidative DNA adducts by dietary intervention and chemoprevention has been demonstrated in preclinical and clinical studies. Protein adducts and DNA adducts in blood constituents or urine may act as useful surrogates for the target organ. Additional information regarding reliability, reproducibility, specificity, and confounding variables are required at the clinical level to validate adducts as suitable biomarkers of chemoprevention.

“We do not administer antihypertensive drugs to patients in clinical trials without checking their blood pressure, so why should we give antioxidants without checking that they have decreased oxidant status (B. Halliwell, Lancet 2000:355:1179–80)?”

INTRODUCTION
Although epidemiological associations between certain chemicals and human carcinogenesis have been observed since the late 18th century, the study of the interactions of these compounds with cellular constituents began in 1947, when their binding to proteins was recognized by Miller and Miller (1). At that time, most scientists were of the opinion that genetic information resided in proteins, and subsequent studies established covalent binding (adduct formation) of carcinogens such as benzo(a)pyrene to proteins (2). Around the same time, the mutagenic effects of mustard gas were discovered by Auerbach and Robson (3), and it was noted that treatment of DNA with mustard gas altered its UV spectrum (4). After the description of the structure of DNA by Watson and Crick (5) in 1953, the chemical structures of a number of DNA adducts resulting from different carcinogens were characterized in the 1960s. With reference to DNA, it became clear that many mutagens are carcinogens, although not all carcinogens are mutagens. The importance of metabolism in carcinogen activation and detoxification has also been elucidated for certain carcinogens such as aflatoxin B1 (AFB1; Ref. 6). Current thinking is that mutation is a possible consequence of binding of carcinogens to DNA, and it is a necessary step in the carcinogenic process of genotoxic carcinogens.

A major change in the field of carcinogenesis research has occurred in the last 2 decades with the development of analytical methods sensitive enough to detect background damage to DNA in healthy humans. This advance has resulted in the realization that DNA damage and mutation arise from endogenous products of cellular metabolism such as oxidative stress and lipid peroxidation (7). Concomitantly, understanding of the role of endogenous enzymes and their polymorphic expression on the activation of procarcinogens such as AFB1 and on the detoxification of reactive metabolites of procarcinogens has been advanced (6). There currently exist at least six different analytical methods (Table 1) for detecting >20 specific DNA adducts, and the emphasis in this field of research has shifted from discovery/characterization of adducts to understanding their biological significance. This review article aims to relate current knowledge of the biological relevance of adducts to the rapidly expanding specialty of cancer chemoprevention.

The concept of chemoprevention is already well established in the prophylaxis of dental caries, ischemic heart disease, and cerebrovascular disease. Although the potential for the chemoprevention of cancer is a relatively new concept (8), hundreds of studies have been reported and a few efficacious agents have been identified. The most obvious example is tamoxifen, which can now be considered an established agent in...
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The prevention of breast cancer in the United States (9). Another example is the inhibition of the cyclooxygenase enzyme in patients with familial adenomatous polyposis, shown to cause regression of potentially premalignant lesions (10). The potential use of adducts as biomarkers of the efficacy of chemopreventive intervention will be discussed in this article. The measurement of adducts in the cancer chemotherapeutic setting, for example those engendered by cisplatin chemotherapy, will not be considered in this review.

Definitions. Cancer chemoprevention can be defined as the inhibition, retardation, or reversal of carcinogenic processes by chemical means (11). The clinical evaluation of agents for chemopreventive properties can be separated into three phases, as shown in Table 2. Unresolved issues posed by such trials include trial duration, how to assess agent safety, subject selection, and number of subjects enrolled (12).

One of the shortcomings of many clinical chemoprevention studies is the absence of suitable biomarkers of efficacy. Biomarkers reflect biological events that take place between exposure to external or endogenous carcinogens and the subsequent development of cancer, which may be modified by therapeutic intervention. It is important to differentiate these biomarkers of carcinogenesis from risk biomarkers, used to estimate risk for an individual of developing cancer, or tumor markers, used to diagnose or monitor malignancy (Table 3). A biomarker may be a discrete event, such as a genetic mutation, or a quantitative change, such as an increase in cell proliferation. Criteria used to identify and judge the potential efficacy of biomarkers of carcinogenesis have been defined by Einspahr et al. (13) and Pepe et al. (14), summarized and expanded in Table 4. In particular, the relevance of information regarding reliability, reproducibility, sensitivity, and confounding variables is emphasized in these articles (13, 14).

Histological biomarkers, currently regarded as the gold standard for a single biomarker used in isolation, may not offer sufficient sensitivity to estimate the risk of premalignancy or early malignancy progressing. For example, it is estimated that <5% of colorectal adenomas become malignant and may take 5–10 years to transform; current histological indices are poor predictors of such transformation (15). Complementary or alternative classes of biomarkers include DNA and protein adducts, which may enhance our ability to study separate molecular and

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<th>Disadvantages</th>
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<td>$^{32}$P-postlabelling</td>
<td>Sensitivity, small amount of DNA</td>
<td>Labor intensive, different labelling efficiencies</td>
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<td>Immunoassays</td>
<td>Sensitivity, small amount of DNA, high throughput</td>
<td>Potential for lack of specificity, dependency on quality of antibody</td>
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<td>Gas or liquid chromatography-mass spectrometry</td>
<td>Specificity</td>
<td>Expensive equipment, large amount of DNA</td>
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<td>Gel electrophoresis or HPLC with fluorescence detection</td>
<td>Specificity and sensitivity</td>
<td>Limited to certain adducts, large amount of DNA</td>
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<td>Electrochemical detection</td>
<td>Sensitivity, inexpensive</td>
<td>Limited to certain adducts</td>
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<td>Atomic absorbance</td>
<td>Sensitivity and specificity</td>
<td>Limited to certain adducts</td>
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<th>Phase</th>
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<tr>
<td>I</td>
<td>15–30</td>
<td>Small, short-term pilot study with rapid dose escalation if no toxicity observed</td>
<td>1. Pharmacokinetics</td>
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<td>Ia</td>
<td>30–60</td>
<td>Small, short-term dose de-escalation study</td>
<td>2. Preliminary data on biomarkers of efficacy</td>
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<td>Iib</td>
<td>500–1500</td>
<td>Larger, medium-term randomised study in high-risk individuals</td>
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<td>III</td>
<td>Several thousand</td>
<td>Large, long-term randomised study with carefully defined risk levels or stratification</td>
<td>Response in validated biomarkers of efficacy</td>
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<td>1. Response in biomarkers</td>
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<td>2. Cancer incidence and mortality</td>
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<th>Type of biomarker</th>
<th>Stage of disease</th>
<th>Description</th>
<th>Application</th>
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<tr>
<td>Risk biomarker</td>
<td>No premalignant or malignant disease detectable</td>
<td>Genetic predisposition, past medical history, lifestyle factors, exposure, cellular abnormalities</td>
<td>Useful to molecular epidemiologists to assess risk of developing cancer</td>
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<td>Biomarkers of chemopreventive intervention</td>
<td>Initiation, promotion, progression</td>
<td>Biological alterations representing the early and intermediate stages of carcinogenesis, which may show alteration by chemopreventive agents</td>
<td>Identification of carcinogenesis and chemoprevention</td>
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<td>Tumor markers</td>
<td>Established neoplasia, metastasis</td>
<td>Elevated levels may be associated with particular cancers</td>
<td>Diagnosis and treatment (of primary condition and relapse)</td>
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biochemical pathways within a cell. An example (see Fig. 1) is provided by a pyrimidopurinone adduct of deoxyguanosine (M₁ G) that may arise from lipid peroxidation, cyclooxygenase catalysis, or oxidative DNA damage, all processes linked with colorectal carcinogenesis (16).

Adducts arise from the chemical modification of bases in DNA or amino acids in proteins by toxic chemicals. Modifications of DNA bases may be small, e.g., oxidized DNA bases (7), or bulky, e.g., covalent interaction between tamoxifen and deoxyguanosine (17). The existence of DNA repair pathways that have evolved to remove adducts arising from endogenous mutagens may be considered strong evidence in favor of their biological relevance to evolution (7).

Techniques for Measurement of Adducts. Analytical methods have developed to such an extent that scientists are now capable of detecting DNA and protein damage in normal tissues from healthy individuals. Some of the methods available for measuring DNA adducts are listed in Table 1 and discussed in more detail below.

3²P-Postlabeling. 3²P-Postlabeling is the most widely applicable and sensitive procedure for the analysis of DNA adducts. The procedure involves the enzymic digestion of DNA to 5'-mononucleotides, enrichment of the adduct, labeling with T4 polynucleotide kinase using [γ-³²P]ATP, and chromatographic separation of the labeled adducts. The latter may be achieved using two-dimensional thin layer chromatography or high pressure liquid chromatography (HPLC). Enrichment (see below) is a key part of the procedure, because this partially purifies the adduct from a vast excess of normal nucleotides. Enrichment is normally achieved using 1-butanol extraction or nuclease P1 digestion (which dephosphorylates normal nucleotides and, thus, prevents them being substrates for the 3²P-phosphorylation). The sensitivity of ³²P-postlabeling reaches adduct frequencies as low as 1 in 10⁹ nucleotides, and it is capable of detecting a very wide variety of lesions, although the procedure is quite labor intensive and requires a dedicated laboratory. Normally, 1–5 µg DNA is needed for analysis. The disadvantage of ³²P-postlabeling is that the chemical identity of the adduct being detected is often not easily identified. The use of standards of predicted adducts is recommended, because, after postlabeling, these may be chromatographically compared with unknown lesions to help the structural interpretation of the latter. Standards also improve the accuracy of quantitation, because they may be used to determine the efficiency of the enhancement and postlabeling procedures. The sensitivity of ³²P-postlabeling may be improved by the use of more selective enhancement procedures or better chromatographic separation of postlabelled adducts, but it is ultimately governed by the specific activity of the [γ-³²P]ATP used for labeling, of which the potential for increase is limited.

Mass Spectrometry. In contrast to ³²P-postlabeling, the sensitivity of mass spectrometry (MS) has increased greatly over recent years, and additional increases are expected. Initially the most sensitive techniques for detection of adducts relied on the use of gas chromatograph-MS (GC-MS), using for example negative chemical ionization of the molecule, and normally derivatization of the adduct was necessary to make it volatile for the chromatography. However, the discovery of electrospray ionization allowed the direct coupling of HPLC columns to mass spectrometers (liquid chromatography-MS) and the sensitive detection of polar compounds without derivatization. The improving sensitivity of liquid chromatography-MS suggests that it may become the method of choice in the future. The particular advantages of GC- or liquid chromatography-mass spectral techniques are that the nature of the chemical being analyzed is known with certainty and that highly accurate quantitation may be carried out by the use of internal standards, preferably labeled with stable isotope. Relatively large amounts of DNA (up to 100 µg) are currently needed for analysis. Over the coming years, the use of capillary liquid chromatography coupled with nanospray MS should result in microgram quantities being sufficient for structural and quantitative analysis. Adduct levels down to 1/10⁸ nucleotides are currently detectable by MS (18).

Immunochromatography Techniques. Monoclonal or polyclonal antibodies are available for some DNA adducts, and immunoassay techniques have been developed for these using standard procedures, e.g., radioimmunoassay, ELISA. The amount of DNA required for this may be similar to that used for

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<td>Variability of expression during carcinogenesis</td>
<td>• Change corresponds to carcinogenic progression</td>
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<tr>
<td>Ability to be detected in target tissue</td>
<td>• Relatively short latency</td>
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<td>Association with development of cancer</td>
<td>• Accessible for multiple biopsies</td>
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<td>Adequate validation and quality control</td>
<td>• Mechanism-based</td>
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<td>• Potential for modification</td>
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<td>• Ability to detect harm as well as benefit</td>
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Table 4 Summary of criteria used to judge the potential efficacy of biomarkers of carcinogenesis with special reference to their value in the chemoprevention of cancer (modified from Refs. 12–14).
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MS techniques, and the immunoassays can be of similar sensitivity to MS. Normally the DNA is digested before immunoassay, but detection of some adducts in intact DNA and immunohistochemistry have also been shown to be possible. A major advance in immunohistochemical detection of adducts was the development of immunoslot/dot blot assays, which only require a small amount of DNA (e.g., 1 μg) which are amenable to high-throughput screening. In brief, adducted single strand DNA is bound to a nitrocellulose filter, treated with an antibody to the adduct, and then treated with a second antibody bearing a light generating system. Sensitivities of these assays are high, e.g., for the M₄G adduct, 2.5 adducts/10⁸ bases (19).

There are a number of other extremely important applications of antibodies to adducts. One involves the preparation of immunoaffinity columns for purification of adducts from complex mixtures before analysis by MS or ³²P-postlabeling. Another is an ultrasensitive assay for measuring DNA base damage that couples immunohistochemical recognition with capillary electrophoresis and laser-induced fluorescence detection. For example, the thymine glycol adduct is highly immunogenic, and antibodies against this adduct have been raised and used to produce an assay with a detection limit of 3 × 10⁻²¹ moles of adduct, an improvement of 4–5 orders of magnitude over current methods (20). Unfortunately, this assay has not been used widely on account of the technical difficulty of the method. In contrast, the availability of immunohistochemical techniques for fixed tissues has resulted in the development of methods for the detection of M₄G and 8-oxo-7,8-dihydro-2'-deoxyguanosine (8-oxo-dG) using antibodies (Fig. 1; Refs. 21, 22). In particular, the ability to detect M₄G adduct levels in oral mucosal cells holds appeal, because buccal smears represent an easy and relatively risk-free way of obtaining tissue samples for biomarker measurement in clinical trials (21).

Sample Workup. The measurement of 8-oxo-guanine adducts has generated debate regarding the potential for artificial formation of adducts during the isolation of DNA, derivatization for MS, or other aspects of the workup of samples. It has been shown, for example, that 8-oxo-dG can be generated from oxidation of unmodified guanine bases present in DNA during the silylation reactions used to produce volatile derivatives for GC-MS (23). The importance of keeping samples as cool as possible (e.g., during centrifugation) and minimizing the content of transition metal ions (e.g., through use of deionized water sources) have been recognized for some time. It is now regarded as essential to incorporate a prepurification step to remove unmodified deoxynucleosides or bases before analysis for oxidized bases, particularly when small amounts of DNA are analyzed. Trials not using freshly isolated tissues should also consider the possible effects of storage conditions, e.g., light and temperature.

Current controversies regarding the potential for artifact formation during the detection of endogenous adducts represent the major stumbling block in the advancement of adducts as biomarkers of chemoprevention. The development of new validated techniques (24) has suggested that earlier studies were subject to artifact generation, particularly the detection of 8-oxo-dG by GC-MS. Newer methods are detecting significantly lower levels of adducts than many of the previous studies (24). The lack of control DNA from unexposed animals or humans may mean that these controversies may never be fully resolved.

Adduct Enrichment. Digestion of adducted DNA produces a mixture of adducts together with a very large excess of normal nucleotides (e.g., 10⁶-fold). Enrichment (partial purification) of the adducts is normally an essential part of the analytical procedure. The most commonly used procedures are 1-butanol extraction (which is appropriate, for example, for aromatic amine adducts) or nuclease P1 digestion (suitable for many high molecular weight adducts). HPLC may also be used for adduct purification before ³²P-postlabeling. If an antibody is available, immunoaffinity purification is an appropriate enhancement technique, dependent on the specificity of the antibody. Mass spectral quantitation of adducts involves chromatography of high resolving power to separate the adduct, but additional enrichment may be needed, as exemplified by a recently developed procedure for detecting 8-oxo-2′-deoxyguanosine, which uses an immunoaffinity column to remove normal nucleosides from the analytical mixture before liquid chromatography-MS (18).

Protein Adducts. Protein adducts, which are not currently known to be involved in the mechanism of carcinogenesis, are not repaired. Therefore, protein adducts form a stable repository of accumulated exposure to carcinogens over the lifetime of the protein. Globin and albumin are favored as proteins in which to measure adducts because of their ready availability from blood and their long lifetime. MS is the procedure most commonly used for protein adduct analysis (25), although immunoassay has been effectively used for detection of some adducts, e.g., the aflatoxin B₁-lysine adduct in albumin, and the NH₂-terminal valine adduct of ethylene oxide in globin (26, 27). NH₂-terminal valine adducts of a wide variety of low molecular weight genotoxins have been quantitated by GC-MS, after a modified Edman degradation technique, and this has led to the detection of environmental, dietary, occupational, and endogenous sources of these adducts (25).

Adducts as Biomarkers of Carcinogenesis and Chemoprevention

Molecular Epidemiology. The field of molecular epidemiology seeks to identify risk of developing cancer based on individual exposures and inherited susceptibilities to cancer (28); biomarkers developed in this field are likely to be useful in the chemoprevention of cancer. The term “molecular epidemiology” was coined by Perera and Weinstein (29) in 1982 as “an approach in which advanced laboratory methods are used in combination with analytical epidemiology to identify at the biochemical or molecular level specific exogenous agents and/or host factors that play a role in human cancer causation.” Continual method development has taken place over the last 2 decades, initially mostly directed toward DNA and protein adduct analysis and more recently toward genotype and gene expression analysis. Among the achievements of molecular epidemiology over this period, in addition to its widespread use for monitoring occupational, environmental, and medicinal exposures to genotoxins, are the definitive association of exposure to AFB₁ to hepatocarcinogenesis by DNA adduct measurements (see below), the discovery of endogenous sources of exposure to carcinogenic substances by DNA and protein adducts measure-
ments (e.g., adducts to ethylene oxide, malondialdehyde, and etheno adducts), and the discovery of unknown sources of exogenous exposure to genotoxic substances (e.g., acrylamide from food found from protein adduct measurements and heterocyclic aromatic amines from food found from DNA adduct measurements).

Recent technical advances in deriving data from genomic or proteomic studies holds great promise for the rapid identification of polymorphic variants or changes in gene expression that influence susceptibility to and response to exogenous and endogenous carcinogens. This explosion in data leads to a major bioinformatic challenge for the interpretation of results and determination of the certainty of such observations. Another rising matter of concern relates to the ethical considerations of such experiments, i.e., how to make correct use of genetic data that may lead to identification of susceptibility of healthy individuals to develop disease. Contemporary accessibility of data on susceptibility, environmental exposure, internal dose, molecular dose, effects in preclinical models, and clinical disease makes translational science (i.e., advancement from preclinical models to clinical trials) a feasible prospect for a biomarker. It also emphasizes the concept of overlap between different classes of biomarker, resulting in a continuum first suggested by Perera and Weinstein in 1982 and modified in subsequent papers (28–30).

**General Features of Carcinogenesis.** Carcinogenesis is a process that results from the accumulation of multiple genetic mutations, resulting in alterations in cellular molecules. Because DNA is efficiently repaired, only a small proportion of total DNA damage results in permanent mutation. Although genetic events during carcinogenesis have traditionally been separated into three phases, initiation, promotion, and progression, this temporal sequence is somewhat misleading, because accumulated changes do not necessarily occur in the same order in different cells. Historically, the initiation phase has been associated with carcinogen binding and damage to DNA. In reality, DNA adduct levels may be relevant to chemoprevention before this stage (i.e., in terms of background levels in normal, healthy individuals) as risk biomarkers or in subsequent phases of carcinogenesis as biomarkers of chemopreventive intervention.

Once a cell has acquired sufficient genetic mutations to exhibit the cancer phenotype, it may act as a progenitor cell for clonal evolution of malignant cells. These cells are characterized by the macroscopic and microscopic changes used to diagnose cancer. It is increasingly recognized that epigenetic phenomena, such as hypermethylation of promoter sequences that control the expression of oncogenes and tumor suppressor genes, play an important role in the clinical significance of abnormal genes. Potential links between oxidative DNA adducts in promoter sequences and levels of binding of transcription factors activator protein 1, Sp1, and nuclear factor-κB have been established in studies using oligonucleotide substrates and recombinant proteins.

**Aflatoxin B1 and Hepatocellular Carcinoma.** Currently the most detailed epidemiological description linking a certain DNA adduct to development of a certain cancer exists for AFB1. Hepatocellular carcinoma is one of the most common cancers worldwide and the leading cause of death in some parts of China and Africa, where chronic infection with hepatitis B and exposure to aflatoxins in the diet are considered the main etiological factors (33). Aflatoxin B1 was originally discovered after poisoning of turkeys in Britain with mold-contaminated peanut meal. The main adduct formed by AFB1 with DNA in preclinical models is called AFB1-Gua (Fig. 2A) and has been identified as 8,9-dihydro-8-(N7-guanyl)-9-hydroxy-aflatoxin B1 (34). When this adduct undergoes ring opening under physiological conditions (Fig. 2B), it results in 8,9-dihydro-8-(N5-formyl-2,5,6-triamino-4-oxopyrimidin N5-yl)-9-hydroxy-aflatoxin B1 (35). These two adducts account for >95% of aflatoxin B1 residues bound to DNA and correlate well with exposure to aflatoxin B1 in vivo (36, 37).

The first report of the detection of AFB1-Gua in human urine samples was in an area of Kenya with a high incidence of liver cancer, where the diet was known to be contaminated with aflatoxin B1 (38). The subsequent development of monoclonal antibody-based immunoaffinity columns for rapid purification of samples before fluorescence spectrophotometry has allowed analysis of large numbers of samples in a relatively short time span. Several studies using this technique have correlated risk of developing hepatocellular carcinoma with urinary levels of AFB1-Gua (reviewed in Ref. 36). In particular, a cohort of 18,244 men residing in Shanghai, China, was followed for almost 70,000 person-years to identify 55 cases of incident hepatocellular carcinoma (39). Highly significant associations were observed between hepatocellular carcinoma risk and the presence of urinary aflatoxins and their metabolic products, including AFB1-Gua, as well as serum hepatitis B surface antigen positivity. Risk was particularly elevated for individuals who were hepatitis B surface antigen positive and who also had aflatoxins detectable in their urine.

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Fig. 2 Chemical structures of AFB1-Gua (A) demonstrating ring opening under physiological conditions (B). dR represents 2’-deoxyribose. For details of biological relevance, see text.
One particularly interesting angle on aflatoxin B1 relevance to mutagenesis is the critical balance between carcinogen activation and detoxification in determining adduct levels and risk for an individual of developing cancer (6). The characterization of AFB1-Gua in 1977 led to the hypothesis that the epoxide of aflatoxin B1 was the main electrophilic product involved in reactions with macromolecules (34, 40). Indeed, the exo isomer of aflatoxin B1 epoxide, formed by human cytochrome P450 3A4, is particularly genotoxic (41, 42). In a recent study, higher levels of this cytochrome P450 isozyme have been found in liver tissue from aflatoxin B1-exposed cases of hepatocellular carcinoma than controls (43). The balance between activating and detoxifying enzymes may be influenced by chemopreventive agents such as oltipraz (see below) or indole-3-carbinol, which induces both cytochrome P450 and detoxification enzymes and prevents aflatoxin B1–induced hepatocarcinogenesis in rodents (44). The relevance of polymorphic enzyme expression to mutagenesis emphasizes the importance of considering the whole organism and the continuum of biomarkers (see above).

8-Oxo-7,8-Dihydro-2′-Deoxyguanosine and Antioxidant Agents. Reactive oxygen species, such as superoxide anions, hydrogen peroxide, and hydroxyl radicals, are thought to be involved in carcinogenesis and have been shown to attack both DNA bases and the deoxyriboseyl backbone of DNA (23). The hydroxyl radical is particularly reactive and can add to DNA bases or abstract hydrogen atoms from DNA (45). Hydrogen peroxide and peroxynitrite, on the other hand, represent more diffusible oxyradicals that may be better placed to reach the nucleus and react with DNA within it (46).

The most extensively studied oxidized DNA product is 8-oxo-dG (47). This adduct has been shown to cause miscoding by DNA polymerase in vitro, it has been associated with mutagenesis in bacterial and mammalian cells, and it also induces GC to TA transversions after replication in human DNA, lesions seen frequently in oncogenes and tumor suppressor genes (48–50). Recent studies using fragments of the c-Ha-ras gene, an oncogene activated in many human cancers, have discovered complex biochemical events involving DNA polymerases leading to strong activation potential of the oncogene when it contains 8-oxo-dG adducts in a mutational hotspot (51). The outcome of such damage in vitro is misreplication, inhibition of replication, or mutagenesis described as “action-at-a-distance.” DNA containing 8-oxo-dG can also be formed by misincorporation of 8-oxo-dGTP via DNA polymerases (52). Reports of levels of 8-oxo-guanine or 8-oxo-dG in human tissues range from one adduct in 10⁷ nucleotides to one adduct in 10⁸ nucleotides. Higher levels of adducts detected in earlier studies may be attributable to artifactual generation of oxidized bases during workup (see above). Other adducts arising from oxidative stress that have been associated with site-specific mutagenesis include 8-oxo-adenine, M₇G, thymine glycol, 5-hydroxyuracil, and uracil glycol (7, 20, 53).

With regard to the potential for cancer chemoprevention, scavenging activated oxygen species is a cellular mechanism displayed by many diet-derived agents such as flavonoids, vitamin E, and isothiocyanates (reviewed in Ref. 11). Such agents influence multiple targets of intracellular signaling pathways, which may be aberrant in malignant cells. In particular, selective inhibition of cyclooxygenase-2 (see above) with agents such as celecoxib (10) has emerged into clinical practice. In addition to measures of the efficacy of the inhibition of cyclooxygenase-2 activity (54), the measurement of other biomarkers of efficacy relevant to the catalytic products of this isozyme and to carcinogenesis, such as 8-oxo-dG or M₇G adduct levels (16, 55), may be considered in the clinical setting. Similarly, selective interference with other cellular pathways relevant to chemoprevention, such as epidermal growth factor receptor pathways, may influence 8-oxo-dG adduct levels (56).

Exposure to both UV and UVB light causes DNA damage within skin cells, both directly and indirectly, by the formation of reactive oxygen species and other radicals (reviewed in Ref. 57). The induction of 8-oxo-dG by UVB has been shown to occur in normal human skin keratinocytes at doses of radiation relevant to normal sun exposure, an effect attenuated by antioxidants such as ascorbic acid added to cultures in vitro (58). Strand breaks and base lesions are commonly detected, particularly small base modifications such as 8-oxo-dG from UV exposure and bulkier lesions such as cyclobutane pyrimidine dimers from UVB exposure (58–60). In skin cells grown in vitro, vitamins C and E, catalase, carotenoids, glutathione, and flavonoids have been shown to protect against certain types of UV-induced DNA damage (reviewed in Ref. 59).

Oxidative DNA Adducts and Prostate Carcinogenesis. A series of reports emanating from the laboratory of Malins et al. (61–64) have shown that modifications detected by Fourier transform-IR spectroscopy, a technique for studying structural alterations in DNA, which permits analysis of spatial locations, cluster sizes, or both, can be linked to prostate carcinogenesis in animals and humans. The alterations detected during ageing and carcinogenesis in the human prostate most strongly correlate with oxidative DNA damage (64), and the same team has also studied the ratio of mutagenic 8-oxo-dG to putatively nonmutagenic formamidopyrimidine lesions of adenine and guanine by GC-MS to demonstrate that their technique has high sensitivity and specificity for detecting oxidative DNA damage (63). Although complex modeling is required for analysis of the data and the levels detected may be prone to artifactual adduct generation due to the methodology used, as little as 20 μg of DNA from prostate tissue is sufficient for analysis, and the authors have even suggested biomarker thresholds for chemoprevention studies based on levels in normal human tissue at various ages of life (63, 64). Apart from adequate validation and quality controls, which have not yet been performed for this biomarker in the clinical setting, the detection of oxidative DNA damage in prostate carcinogenesis appears to meet the primary criteria for a suitable biomarker of chemopreventive intervention defined in Table 4. It should be re-emphasized from the earlier discussion that levels of 8-oxo-dG detected by GC-MS are currently subject to scepticism on account of the potential for artifactual adduct formation during workup.

With regard to the secondary criteria defined in Table 4, the detection of oxidative DNA damage in prostate carcinogenesis is an attractive candidate as a biomarker of chemopreventive intervention. In particular, dietary antioxidant intervention has resulted in significant changes in adduct levels detected in the hind legs of rodents (65) and in the canine prostate (66). Contemporary translation of these studies to Phase I clinical trials is discussed below.
M1G Adducts. Initial interest in M1G adducts arose from the knowledge that malondialdehyde is generated during lipid peroxidation and the potential link between lipid peroxidation and carcinogenesis. M1G adducts have been detected in breast, liver, white blood cells (WBC), pancreas, and colon mucosa from healthy humans at levels ranging from 1 to >120 adducts per 10^8 nucleotides (7). In an early clinical project, Wang et al. (67) discovered that M1G adduct levels in normal breast tissue from patients with breast cancer were significantly higher than M1G adduct levels in normal breast tissue from control subjects without breast cancer. Although age and body mass were not found to significantly influence adduct levels, control subjects were not matched for these variables, nor for smoking status or previous detection of a benzo(a)pyrene adduct in breast tissue, both of which have been found to influence M1G adduct levels significantly. Although cause and effect cannot be differentiated from such observations, this study provided the first clinical indication that DNA adduct levels may differ in apparently normal tissue from patients with cancer compared with patients without cancer. This concept arises from the knowledge that in rat liver, the limit of detection of DNA adducts formed by toremifene and styrene can be well below the limit of detection of increased tumor incidence (68).

More recently, a similar finding has been published by Leuratti et al. (69) in patients with colorectal adenomas considered precursor lesions to adenocarcinoma. Biopsies from the histologically normal colon mucosa of 162 healthy volunteers were analyzed by immunoslot blot assay, demonstrating a trend toward higher M1G adduct levels in individuals found to have adenomas elsewhere in the colon at the time of biopsy, especially in subset analysis of those subjects with the highest levels of adducts. Controls were age, sex, and geographically matched, and detailed information was available regarding diet, smoking status, weight, height, and body mass index. Interesting associations were observed regarding diet, alcohol consumption, height, and age.

Predisposition to cancer can also result from infection; for example, Helicobacter pylori infection in humans is association with the development of gastric adenocarcinoma and lymphoma (70). A similar infection caused by Helicobacter hepaticus has been associated with chronic hepatitis and hepatic carcinogenesis in mice (71). On account of the role of lipid peroxidation and reactive oxygen species in such infection, levels of M1G and 8-oxo-dG adducts in liver DNA from infected and control mice have been studied using immunochemical assays (72). Levels of M1G adducts were age dependent and lobe specific, with H. hepaticus infection associated with higher levels of M1G than controls. Such findings have been translated into clinical projects studying biopsy tissue from patients with H. pylori infection using the same assay for M1G. Gastric mucosal biopsies were collected from 124 patients attending for routine endoscopy to look for H. pylori infection (73). No association was found between the presence of infection and the level of M1G adducts.

It has now known that malondialdehyde is not the principal route of M1G adduct formation. In the same study (73), regression analysis of the gastric biopsy samples from 39 individuals with normal gastric histology and no evidence of H. pylori infection demonstrated that a 200% increase in M1G burden was reflected by an average increase in M1G of only 23%. Additional clinical evidence has been offered in a project undertaken by Kadlubar et al. studying parameters of oxidative stress and oxidative DNA damage in pancreatic tissue from 30 volunteers (74). A significant correlation between levels of M1G and 8-oxo-dG was observed, but there was no suggestion that malondialdehyde contributed significantly to the M1G burden. A principal route of M1G formation appears to involve base propenals, particularly those derived from the direct oxidation of deoxyribose (53).

Despite the appeal of high throughput analysis of small amounts of DNA, the detection of M1G by immunochemical techniques must be approached with some degree of caution. The M1G adduct is unstable and can exist in ring-open or ring-closed conformations (7). It is not currently known whether the antibody used in the studies described above detects one or both forms. Recent studies showing very low levels of M1G in human liver tissue (24) suggest that earlier studies may have been influenced by artifactual adduct formation or lack of specificity in detection.

Etheno Adducts. First described in 1971, etheno adducts in DNA (Fig. 3) are generated by the reaction of bases in DNA with lipid peroxidation products or xenobiotics such as vinyl chloride or urethane (75, 76). Although differentials in the mutagenic potency of etheno adducts have been demonstrated in different model systems, there is extensive evidence to support the hypothesis that etheno adducts are mutagenic (reviewed in Ref. 7). Iron or copper overload, carbon tetrachloride treatment, or ethanol administration can increase hepatic levels of etheno adducts in rats (76), suggesting the importance of lipid peroxidation and oxygen radicals in generation of the adducts. In an elegant example of translational science, Nair et al. (77) detected levels of etheno adducts in hepatic tissue from patients with Wilson’s Disease or primary hemochromatosis about three times higher than control samples from individuals without genetic metal storage diseases. Patients with hemochromatosis...
have a predisposition for developing hepatocellular carcinoma. Similar to the studies of M1G in the context of chronic infection, which may be premalignant (see above), there exists considerable interest in the potential formation of etheno adducts by chronic infection via reactive nitrogen species (78).

Adducts as Biomarkers of Intervention in Clinical Trials

Dietary Modification. The first demonstration of the potential that exists for modification of endogenously derived DNA adduct levels by dietary intervention in humans was reported by Fang et al. (79). Because dietary polyunsaturated fatty acids are the source of polyunsaturated membrane lipids in the body, which are the substrates for both lipid peroxidation and prostaglandin production, they postulated that the levels of formation of DNA adducts by malondialdehyde are affected by dietary fatty acid composition. They recruited 59 healthy individuals who were considered “homogenous with respect to diet,” of whom half were fed a diet rich in polyunsaturated fatty acids and the other half were fed a diet rich in monounsaturated fatty acids for 25 days. Levels of M1G adducts were measured in total WBCs by 32P-postlabeling using reversed-phase HPLC. Despite significant interindividual variation in adduct levels, the average M1G level in the polyunsaturated fatty acids-fed group was 3.6-fold higher than that of the group consuming the monounsaturate-rich diet. As demonstrated in a previous feasibility study measuring adducts by 32P-postlabeling (80), female volunteers were found to have WBC DNA adduct levels higher than those of males. This discrepancy between genders may be even more marked for etheno adducts (1, N-6-ethenoxyadenosine and 3, N-4-ethenoxyxycytidine) in human WBCs. In a study confirming the elevation of WBC M1G adducts by the addition of polyunsaturated fatty acids to the diet, etheno adducts were on average found to be 40 times higher in females than in males (81). Estrogens have been shown to generate aldehydic DNA lesions in calf thymus DNA in vitro (82) and M1G adducts in the kidneys of hamsters in vivo (83).

Although the investigators in the studies described above did not isolate participants to monitor dietary intake, subsequent studies have demonstrated the potential that exists for modification of WBC M1G levels in individuals on strictly controlled high protein diets by the addition of vegetables and tea for 15 days (84). Collectively these studies demonstrate the significant interindividual and intraindividual variation that exists in the measurement of endogenously derived DNA adducts, which must be considered carefully in the power calculations while planning clinical trials.

Development of Surrogate Biomarkers. The development of biomarkers for the chemoprevention of cancer in tissues not readily accessible for repetitive analysis requires pilot studies to find potential “surrogate” measurements in other organs. The concept of altering the “oxidant status” of an individual in many tissues simultaneously by chemopreventive intervention is particularly pertinent to the measurement of oxidative DNA adducts as potential biomarkers of intervention. A correlation has been observed between 8-oxo-dG levels in blood leukocytes and liver tissue in patients with chronic hepatitis B and C infections (85), which are both associated with an increased incidence of hepatocellular carcinoma. A preclinical link has been established between the ability of green tea to prevent increases in rodent hepatic 8-oxo-dG levels and its ability to prevent carcinogenesis in the biliary tract, a process induced by the environmental pollutant pentachlorophenol (86). In a preclinical study of dietary selenium designed in parallel with large-scale clinical trials of this agent in the chemoprevention of prostate cancer in men, Waters et al. (66) fed sexually intact elderly beagle dogs 3 or 6 μg/kg/day selenium daily for 7 months. DNA damage in prostate tissue and peripheral blood lymphocytes was studied using the alkaline comet (single-cell gel electrophoresis) assay, and decreases were demonstrated in both tissues. In a notable study of dietary intervention, the benefit of incorporating measurement of adduct levels in a surrogate tissue has been demonstrated. Because the risk of prostate cancer appears to be lower in men reporting higher consumption of tomato products, which contain high levels of the antioxidant lycopene, Chen et al. (87) investigated the effect of a tomato sauce-based entrée as a dietary intervention in 32 men with localized prostate adenocarcinoma for 3 weeks. In addition to serum and prostate lycopene concentrations, levels of 8-oxo-dG were measured by HPLC with electrochemical detection in blood leukocytes and prostate tissue before and after the intervention. Significant decreases in adduct levels were observed in leukocytes and prostate tissue as a result of the intervention. A particularly interesting correlation was observed between adduct levels in blood leukocytes and prostate tissue (r = 0.51; P = 0.005). Decrease in 8-oxo-dG levels in the blood cells was of the order of 20%, as observed in a previous placebo-controlled pilot study of 1 week of tomato oleoresin intake on lymphocyte 8-oxo-dG levels in healthy volunteers (88).

Although the biological relevance of leukocyte DNA adduct levels is not fully understood per se, they may represent a useful indicator of oxidant status in the whole organism, with clear potential for modification by dietary or chemopreventive intervention. New techniques must be validated in pilot clinical studies to assess inter- and intraindividual variability, reproducibility, and confounding variables before measurement of potential correlations. Such validation is vital to understanding the significance of preliminary findings regarding adduct levels as indicators of oxidant status. Examples of pilot studies providing useful information for biomarker validation in different subject populations are varied: a correlative study in healthy adult volunteers measuring 8-oxo-dG levels in urine by ELISA and 8-oxo-dG levels in lymphocytes by HPLC (89); a study of lymphocyte 8-oxo-dG levels in of infants comparing the antioxidant effects of formula feed versus maternal breast milk (90); measurement of levels of lymphocyte and urinary 8-oxo-dG levels by ELISA in patients with chronic renal failure (91); and detection of urinary 8-oxo-dG in men to look for potential correlations with serum β-carotene levels (92). Unfortunately, many studies have been performed without distinguishing subtypes of circulating WBCs; the life span of neutrophils is much shorter than that of monocytes.

Aflatoxin B1 Adducts. Some adducts are formed exclusively from exogenous carcinogens. As discussed above, the potent hepatocarcinogen aflatoxin B1 forms adducts with human DNA and proteins under physiological conditions. Because the extent of aflatoxin contamination in foods is a function of the ecology of molds and is not completely preventable, cancer
chemoprevention strategies are being developed. Although more than a score of different agents inhibit aflatoxin-induced hepatocarcinogenesis in preclinical models, thus far only two agents, oltipraz and chlorophyllin, have been administered to healthy adults in Phase I/II clinical trials with measurement of protein and DNA adducts respectively. Oltipraz is 4-methyl-5-(N-2-pyrazinyl)-1,2-dithiole-3-thione. Chlorophyllins are water-soluble antioxidant salts derived from the human diet, which form tight molecular complexes with carcinogens such as aflatoxin B₁.

As discussed above, studies in animals and humans have established serum aflatoxin-albumin adduct levels as reproducible biomarkers of exposure to aflatoxin B₁. In a chemical rodent model, decreases in such adducts associated with oltipraz treatment appear to have predictive value with regard to the reduction in risk of developing hepatocellular carcinoma (93). In 1995, a Phase IIa chemoprevention trial of oltipraz was performed in 234 adults from Qidong, People’s Republic of China. Measurement of potential changes in aflatoxin-albumin adducts as a result of 16 weeks of treatment with oltipraz was a primary end point of this trial (94). No consistent changes were observed in this end point, and studies of longer-term treatment are ongoing. It is conceivable that 16 weeks is not the optimal time to measure changes on account of the long half-life of albumin adducts (see below). Measurement of the much shorter-lived aflatoxin metabolites was also performed (94), and differences were found between the two dose levels (125 mg once daily or 500 mg weekly). Because the sustained lower dose regime increased Phase II conjugation of aflatoxin more favorably than the intermittent high-dose regime (95) and this mechanism of metabolism is considered favorable for chemoprevention, the former has advanced to Phase IIIb studies.

The adduct formed by the metabolic products of aflatoxin B₁ with N⁷-guanine in human DNA is a biomarker not only of biologically relevant exposure to this potent hepatocarcinogen but also of risk of developing liver cancer (93). Urinary levels of this adduct have been associated with a liver cancer risk at least double that of individuals without detectable traces in urine (39). In contrast to the half-life of ~21 days for aflatoxin-albumin adducts, aflatoxin-N⁷-guanine adducts have a biological half-life of ~8 h, and, therefore, reflect very recent exposure (96). Kensing et al. (97) have suggested that, although strong concordance exists between liver tissue and urinary excreted levels of aflatoxin-N⁷-guanine adducts in experimental models, this biomarker may underestimate the clinical efficacy of a chemopreventive intervention as assessed by tumor burden. In a randomized, double-blind, placebo-controlled chemoprevention trial, 180 healthy adults from Qidong ingested 100 mg of chlorophyllin or a placebo three times daily for 4 months (96). Volunteers were eligible only if aflatoxin-albumin adducts could be detected in their serum at baseline (i.e., 215 of 511 individuals screened). On the basis of knowledge of the pharmacokinetics of the compound, levels of aflatoxin-N⁷-guanine adducts were measured in urine by liquid chromatography-MS after 12 weeks of intervention. Levels of aflatoxin-N⁷-guanine adducts in the urine of those consuming the chlorophyllin thrice daily were 55% lower than levels in samples from those receiving the placebo (P = 0.036). Analysis of other time points (urine samples were collected every 4 weeks) is ongoing. The structure of this Phase II trial provides an elegant example of the positive results that can be obtained with an adequately validated biomarker as the principal end point using an intervention that has been well characterized pharmacologically.

Conclusions

Adducts arise from the chemical modification of bases in DNA or amino acids in proteins by toxic chemicals. Biomarkers of carcinogenesis reflect biological events that take place between exposure to external or endogenous carcinogens and the subsequent development of cancer. Therapeutic intervention for the purpose of cancer chemoprevention may modify these biomarkers. In this article, the potential efficacy of oxidative DNA adducts and aflatoxin DNA and protein adducts as biomarkers of carcinogenesis and chemoprevention has been discussed using criteria defined for phases of biomarker development. Using modern techniques, the sensitivity of adduct detection in histologically normal tissue offers opportunities for the detection of carcinogenesis earlier than routine histological diagnosis. Currently the challenge lies in understanding the biological significance of the levels detected.

Associations have been observed between levels of aflatoxin adducts and the incidence of liver tumors in rodents and humans. No definite link has yet been demonstrated between the absolute levels of oxidative DNA adducts and the risk of developing human cancer. Variability of levels of oxidative DNA adducts between stages of the carcinogenic process (e.g., normal, premalignant, and malignant specimens) has been observed in the human prostate. Using modern analytical techniques such as capillary LC with nanospray MS or immunoassay, adduct levels are quantifiable in tissues that are accessible for multiple biopsies. Oxidative DNA adducts such as 8-oxo-dG and M₂G in blood cells, urine, or oral mucosal cells may act as useful surrogates for the target organ, albeit with consideration of the potential for artifactual adduct generation during sample workup. Measurements of M₂G and 8-oxo-dG adduct levels in pilot clinical trials provide the best current examples of translational research. Potentially favorable modification of adduct levels by dietary intervention and chemoprevention has been demonstrated in preclinical and clinical studies. To establish their role in clinical chemoprevention, additional information regarding reliability, reproducibility, sensitivity, and confounding variables is required to validate adducts as suitable biomarkers.

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Biological Relevance of Adduct Detection to the Chemoprevention of Cancer

Ricky A. Sharma and Peter B. Farmer


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