Carcinogen biomarkers for lung or oral cancer chemoprevention trials

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The potential applicability of specific carcinogen-derived biomarkers in chemoprevention trials against lung and oral cancer is discussed. At present, there are no examples of the use of these biomarkers in chemoprevention trials, but the principle has been established in chemoprevention trials directed at aflatoxin B1-induced liver cancer. Polycyclic aromatic hydrocarbons (PAHs) and tobacco-specific nitrosamines are among the most important carcinogens invoked as causes of lung and oral cancer. Biomarkers that are potentially practical for current application in chemoprevention trials are 7,8-dihydroxy-9,10-epoxy-7,9,10-tetrahydrobenzo[a]pyrene-DNA adducts, as determined by HPLC with fluorescence detection, nitrosamino acids in urine, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol and its glucuronides in urine, nicotine metabolites in urine, and metabolites of cytochrome P450 substrates in urine. Biomarkers that need further development or exploration before application in trials include 7-methylguanine in DNA, tobacco-specific nitrosamine—DNA adducts, acrolein—crotonaldehyde—DNA adducts, PAH—protein adducts, acetahydro—protein adducts, pyrene metabolites in urine and benz[a]pyrene metabolites in urine. Such carcinogen derived biomarkers could be applied in chemoprevention trials to test the hypothesis that chemopreventive agents alter carcinogen metabolic activation and detoxification and, ultimately, risk for cancer.

Introduction
Carcinogen-derived biomarkers are quantifiable compounds that are formed from specific carcinogens. Examples are adducts with DNA, haemoglobin or albumin, and metabolites in blood or urine. No examples have yet been reported of the use of these biomarkers in lung or oral cancer chemoprevention trials in humans. However, they are being effectively employed in studies of liver cancer prevention by oltipraz in individuals with high exposure to aflatoxin B, (Kensler et al., 1997, 1998; Groopman & Kensler, 1999; see also Wild & Turner in this volume). There is every reason to believe that similar strategies can be used in studies of lung and oral cancer. This chapter discusses specific carcinogen-derived biomarkers potentially suitable for application in chemoprevention trials against cancers of the lung and oral cavity in humans.

Carcinogen involvement in lung cancer
Cigarette smoking causes 87% of lung cancer (American Cancer Society, 2000). There are 55 carcinogens in cigarette smoke that have been evaluated by IARC and for which there is sufficient evidence for carcinogenicity in either laboratory animals or humans (Hecht, 1999). Of these, 20 compounds have been found convincingly to induce lung tumours in at least one animal species and have been positively identified in cigarette smoke (Table 1) (Hecht, 1999). The potential contributions of each of these compounds, as well as free radicals and oxidative damage, to lung cancer induction in humans have been evaluated (Hecht, 1999). The evidence is strongest for specific polycyclic aromatic hydrocarbons (PAHs), typified by benzo[a]pyrene (B[a]P) and the tobacco-specific nitrosamine 4-(methylnitrosamino)-1-(3-pyridyl)-1-butane (NNK). Figure 1 presents a scheme linking nicotine addiction and lung cancer via B[a]P, NNK and other carcinogens of cigarette smoke (Hecht, 1999). Nicotine addiction is the reason that people continue to smoke. While nicotine itself is not considered to be carcinogenic, each cigarette contains small doses of B[a]P, NNK and other carcinogens of cigarette smoke (Hecht, 1999). Nicotine addiction is the reason that people continue to smoke. While nicotine itself is not considered to be carcinogenic, each cigarette contains small doses of B[a]P, NNK and other carcinogens. Although each individual dose is small, the overall carcinogen dose in years of smoking is substantial. Most carcinogens in cigarette smoke require metabolic activation to exert their carcinogenic effects via formation of DNA adducts. Several competing detoxification processes protect
<table>
<thead>
<tr>
<th>Carcinogen class</th>
<th>Compound</th>
<th>Amount in mainstream cigarette smoke (ng/cig)</th>
<th>Sidestream/mainstream ratio</th>
<th>Representative lung tumorigencity in species</th>
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*From Hecht (1999)*
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**Figure 1. Scheme linking nicotine addiction and lung cancer via tobacco smoke carcinogens**

against carcinogenesis. The balance between metabolic activation and detoxification is a target for chemopreventive agents. If metabolic activation can be decreased or detoxification increased, then DNA adduct formation should decrease. This should result in a decreased number of permanent mutations in critical regions of oncogenes and tumour-suppressor genes, and a consequent decrease in loss of normal cellular growth control mechanisms, with the ultimate result being decreased cancer incidence.

The biomarkers considered here are those that would specifically relate to changes in metabolic activation and detoxification of lung or oral carcinogens.

**Carcinogen involvement in oral cancer**

Cigarette smoking causes 90% of oral cancer in males and 60% in females (Shopland, 1995). Unlike lung cancer, the risk of oral cancer is multiplicatively enhanced by alcohol consumption (Blot et al., 1996). The mechanistic framework illustrated in Figure 1 can also be applied to oral cancer, although the genetic changes have not been characterized as extensively. There is good evidence from animal studies that PAHs and tobacco-specific nitrosamines such as NNK and N'-nitrosonornicotine (NNN) play a significant role as causes of cancer of the oral cavity (Hecht & Hoffmann, 1989; Hoffmann & Hecht, 1990). Other nitrosamines may be involved, particularly in combination with alcohol intake (Chhabra et al., 1996). Acetaldehyde, the major metabolite of ethanol, and other aldehydes in tobacco smoke may also contribute (Vaca et al., 1998).

Snuff-dipping causes oral cancer (IARC, 1985). Tobacco-specific nitrosamines are the most prevalent strong carcinogens present in snuff and are likely to play a significant role as causative agents in people who use these products (Hecht, 1998). Betel-quid chewing, with tobacco, is the major cause of cancer of the oral cavity in India and other parts of southern Asia. Tobacco-specific nitrosamines, areca-specific nitrosamines and oxidative damage are believed to be causative factors (IARC, 1985; Hecht, 1998; Hoffmann et al., 1994; Bartsch et al., 1999).

**DNA adducts as biomarkers**

**PAHs**

A large number of studies have quantified 'PAH-DNA adducts' by immunochemical methods. These will not be considered further here because they do not quantify specific PAH adducts, but rather measure them as a group (Santella, 1999). Individual PAHs differ widely in carcinogenic activity, limiting the utility of this approach for chemoprevention trials. Similarly, many studies have employed 32P-postlabelling to investigate 'hydrophobic-DNA adducts', some of which are probably PAH-DNA adducts, in humans (Krick et al., 1998). None of these studies has quantified specific PAH-DNA adducts. Consequently, the same limitations apply as to the immunochemical studies.

More specific methods including synchronous fluorescence spectroscopy, phosphorescence, and HPLC with fluorescence detection (HPLC-fluorescence) of released tetraols have been developed for quantification of B[a]P-DNA adducts formed via 7,8-dihydroxy-9,10-epoxy-7,8,9,10-tetrahydroB[a]P

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(BPDE), the major ultimate carcinogen of B[a]P (Kriek et al., 1998). Of these, HPLC-fluorescence analysis of released tetraols has been applied most extensively. BPDE-DNA adducts have been detected in human lung, and relationships to aryl hydrocarbon hydroxylase inducibility and polymorphisms in genes for carcinogen-metabolizing enzymes have been observed (Alexandrov et al., 1992; Rojas et al., 1998, 2000). BPDE-DNA adducts have also been detected in human white blood cells (Rojas et al., 1995). White blood cell DNA would be a potentially useful surrogate for lung DNA, but the relationship of adduct levels to those in the lung has not been extensively investigated.

In mice, BPDE-DNA adduct levels measured by HPLC-fluorescence of released tetraols are decreased in lung and liver by pretreatment with benzyl isothiocyanate (BITC), an inhibitor of B[a]P-induced lung tumorigenesis (Sticha et al., 2000). However, there may be limitations to the application of this assay in chemoprevention trials in humans. In one study of adduct levels in coke-oven workers, the levels in 50% of the samples were below the detection limit (Rojas et al., 1995). Among smokers in the same study, 65% of the samples had undetectable adduct levels. Similar results were obtained in a recent larger investigation; overall 52% of exposed individuals including coke-oven workers and smokers had detectable levels of the adduct (Rojas et al., 2000). Remarkably, adduct levels were detected in 93% of subjects with GSTM1-null genotype, but not in individuals who were GSTM1-positive (Rojas et al., 2000). Therefore, this biomarker may have utility in chemoprevention trials with smokers, provided they are preselected as GSTM1-null. Otherwise, the relatively frequent occurrence of non-detectable adduct levels would impose practical limitations on the use of this biomarker in chemoprevention studies.

Nitrosamines

Acid or enzymatic hydrolysis of DNA isolated from tissues of rodents treated with NNK or NNN releases 4-hydroxy-1-(3-pyridyl)-1-butanone (HPB) (Hecht, 1998). Studies in vitro and in vivo have demonstrated conclusively that this arises via pyridyloxobutylation of DNA (Hecht, 1998). HPB-releasing adducts in DNA can be quantified by gas chromatography/mass spectrometry (GC/MS) (Foiles et al., 1991). In rats, DNA adduct levels in lung cell types are decreased by chronic treatment with phenethyl isothiocyanate (PEITC), consistent with inhibition of NNK-induced lung carcinogenesis by PEITC (Staretz et al., 1997). The GC/MS method has been applied in limited studies in humans. One investigation detected HPB-releasing DNA adducts in the lungs of smokers, while a second was negative (Foiles et al., 1991; Blomeke et al., 1996). There have been no reports on analysis of HPB-releasing DNA adducts in white blood cells, but their levels would be expected to be low, based on the available lung data. This methodology does not appear to have adequate sensitivity at present for application in chemoprevention trials in humans.

Methylation of DNA by nitrosamines results in the formation of O- and 7-methylguanine (O- and 7-mG). These adducts may be produced upon exposure to NNK or other methylating nitrosamines such as N-nitrosodimethylamine. Their levels in human lung have been examined in several studies (Hecht & Tricker, 1999). The average level of O-6-mG is reported to be 27 adducts per $10^8$ nucleotides in peripheral lung. Levels of 7-mdG in lung DNA are reported to range from 2.1 to 42.7 adducts per $10^7$ dG; one study has reported higher levels of these adducts in smokers than in non-smokers. Based on limited data, levels of 7-mdG in lymphocyte and bronchial DNA are correlated (Mustonen et al., 1993). Highly sensitive methods have been developed for detection of O-6-mG in human DNA (Kyrtopoulos, 1998). However, this adduct was non-detectable in 95% of 407 peripheral blood leukocyte DNA samples analysed (EUROGAST Study Group, 1994).

Other DNA adduct biomarkers

Adducts of acrolein and crotonaldehyde have been detected in human leukocyte DNA (Nath et al., 1996). In gingival tissue DNA, levels were higher in smokers than in non-smokers (Nath et al., 1998). This assay has not been tested with exfoliated oral cells. Acetaldehyde-DNA adducts have been detected in peripheral white blood cells of alcohol abusers, but rarely in control subjects (Fang & Vaca, 1997). 8-Oxo-dG is commonly detected in exfoliated oral cells (Yarborough et al., 1996), leukocyte DNA (van Zeeland et al., 1999; Asami et al., 1997) and urine (Prieme et al., 1998). Some conflicting results have been obtained,
perhaps due to methodological difficulties (Collins, 1999). With the use of adequate techniques, 8-oxo-dG measurements could be useful particularly in studies of oral cancer associated with betel quid use, in cases where tobacco is not involved. Nevertheless, the significance of this adduct with respect to carcinogenesis is still unclear.

**Protein adducts as biomarkers**

**PAHs**

Non-specific immunochemical methods have been used to quantify PAH-albumin adducts, with the same limitations as discussed above (Tang et al., 1999). Specific methods have been developed for quantitation of BPDE-haemoglobin and albumin adducts, with detection by GC/MS or fluorescence. In a study of newspaper vendors in high-density traffic areas, 60% of the subjects had detectable BPDE-haemoglobin adduct levels (Pastorelli et al., 1996). BPDE-haemoglobin adducts were detectable in 14% of lung cancer patients, while BPDE-albumin adducts were detectable in 55% (Pastorelli et al., 1998). In 65 employees with no occupational exposure, BPDE-haemoglobin adducts were detectable in 11% of the subjects in summer and 16% in winter (Pastorelli et al., 1999). In another study, levels of these adducts were higher in smokers than in non-smokers and were detectable in all subjects. Adducts of chrysene-1,2-diol,3,4-epoxide were also detected by GC/MS (Melikian et al., 1997). Laser-induced fluorescence detection of BPDE-albumin adducts promises to be highly sensitive, but limited human data are available (Ozbal et al., 1999). The overall utility of BPDE-protein adducts in chemoprevention studies is unclear at present, as there are discordant data with respect to detectability.

**Nitrosamines**

The tobacco-specific nitrosamines NNK and NNN form adducts with haemoglobin by pyridyloxobutylation of globin (Hecht, 1998). Mild base treatment of this haemoglobin releases HPB, which can be reliably quantified by GC/MS. In rats, levels of HPB-releasing haemoglobin adducts, which are esters, correlate with the corresponding pulmonary levels of DNA adducts formed by NNK and are decreased significantly by the chemopreventive agent PEITC (Murphy et al., 1990; Hecht et al., 1996). In initial studies, this adduct was detected in 73% of smokers, but later investigations found detectable levels in only 10-15% of subjects (Carmella et al., 1990; Hecht, Carmella & Murphy, unpublished data). Adduct levels in smokers are generally close to background levels, limiting the utility of this assay as a biomarker in chemoprevention studies (Hecht, 1998). Higher adduct levels have been found in snuff users than in smokers (Hecht, 1998).

Methylation of globin by alkylating nitrosamines does not appear to be useful because of the high background levels resulting from endogenous methylation processes (Tornqvist et al., 1988). Globin ethylation has been investigated only superficially to date (Kautiainen et al., 1989).

**Other protein biomarkers**

Of the compounds considered here, acetaldehyde has attracted the most attention with respect to protein adducts. Acetaldehyde forms stable imidazolidinone adducts by reaction with N-terminal valine (Conduah Birt et al., 1998; de Jersey et al., 1992). Formation of such adducts in human haemoglobin can be monitored by mass spectrometry and may be applicable as a biomarker of acetaldehyde uptake (Conduah Birt et al., 1998). Malondialdehyde-acetaldehyde protein adducts have also been characterized (Kearley et al., 1999). Immunoassay techniques have been employed to quantify acetaldehyde-haemoglobin adducts in alcoholic patients (Lin et al., 1993). Other protein adducts of acetaldehyde have been described, but there are few quantitative studies in humans (de Jersey et al., 1992; IARC, 1999).

**Urinary metabolites as biomarkers**

**PAHs**

Urinary metabolite analysis could be used to test the hypothesis that intervention with a chemopreventive agent alters carcinogen metabolism either by enhancement of detoxification or inhibition of metabolic activation. 1-Hydroxypyrene is the most widely used biomarker of PAH uptake (Jongeneelen, 1997). Levels of 1-hydroxypyrene glucuronide exceed those of free 1-hydroxypyrene (Strickland et al., 1994). Most studies have measured total 1-hydroxypyrene, after hydrolysis of conjugates (Jongeneelen, 1997). Levels of this biomarker increase daily in smokers, intake of charcoal-broiled meat exposure to PAHs (Jongeneelen, 1997;
Sithisaranul et al., 1997; Wu et al., 1998). The effects of chemopreventive agents have not been examined, but presumably in situations of constant exposure, inhibition or induction of cytochrome P450 1A1 could modulate levels of total 1-hydroxy-pyrene. Evidence for this was found in the study of Wu et al. (1998), who observed effects of a CYP1A1 polymorphism on 1-hydroxypyrene levels after adjusting for PAH exposure in coke-oven workers.

We are now developing methods to analyse B[a]P metabolites in human urine, with the ultimate goal of developing B[a]P activation/detoxification metabolite profiles which could be applicable in chemoprevention studies. The first step in this work was development of a method for analysis of r-7,t-8,9,c-10-tetrahydroxy-7,8,9,10-tetrahydro-benzo[a]pyrene (trans-anti-B[a]P-tetraol, the major hydrolysis product of anti-BPDE) in human urine. This metabolite has been detected and quantified by GC/MS in the urine of coke-oven workers, psoriasis patients treated with a coal tar-containing ointment, and cigarette smokers (Simpson et al., 2000). There are also limited published data on urinary metabolite profiles in workers exposed to phenanthrene, chrysene and B[a]P (Grimmer et al., 1997).

Nitrosamines

4-(Methylnitrosoamo)-1-(3-pyridyl)-1-butanol (NNAL) and its glucuronides (NNAL-Gluc) are major urinary metabolites of NNK (Hecht, 1998). These metabolites have been quantified in the urine of smokers, sniff-dippers, ex-smokers, individuals exposed to environmental tobacco smoke and newborns of smoking mothers (Hecht, 1998; Lackmann et al., 1999; Hecht et al., 1999a). They are also sensitive to the effects of chemopreventive agents. In rats treated with PEITC, levels of urinary NNAL plus NNAL-Gluc increased 4-6-fold in tandem with decreased haemoglobin adduct formation (Hecht et al., 1996). These results are consistent with inhibition by PEITC of hepatic NNK metabolism. In smokers who consumed watercress as a source of PEITC, levels of NNAL-Gluc as well as of NNAL plus NNAL-Gluc increased significantly, suggesting similar effects of PEITC in rats and humans (Hecht et al., 1995). In mice treated with indole-3-carbinol, an inhibitor of NNK-induced lung tumorigenesis, levels of urinary NNAL decreased due to increased hepatic clearance of NNK (Morse et al., 1990). Similar effects were observed in smokers treated with indole-3-carbinol (Taioli et al., 1997).

Quantification of NNAL and NNAL-Gluc is a practical method for obtaining information on the effects of chemopreventive agents on NNK metabolism in smokers. NNAL and NNAL-Gluc are readily quantified in the urine of all smokers, providing an excellent biomarker of lung carcinogen uptake (Hecht, 1998, 1999). Provided that there is a specific hypothesis to be tested with respect to the chemopreventive agent employed, this biomarker should be widely applicable in chemoprevention studies.

Endogenous formation of N-nitroso compounds can be monitored by the quantification of urinary nitrosamino acids (Bartsch & Spiegelhalder, 1996). This methodology is highly practical and has been widely applied in studies of endogenous nitrosation and its inhibition, related to cancers of the oral cavity, oesophagus and other sites. Thus, chemopreventive agents which inhibit nitrosation can be evaluated in this way. However, effects on metabolism could not be evaluated since these compounds are in general excreted unchanged.

3-Methyladenine is a compound excreted in the urine that could result in part from metabolic activation of nitrosamines. However, there are multiple sources of urinary 3-methyladenine, including the diet (Fay et al., 1997). In controlled studies, levels of 3-methyladenine in the urine of smokers were elevated. Similar results have been obtained with 3-ethyladenine, but the source of the ethylating agent is unknown (Kopplin et al., 1995; Prevost & Shuker, 1996; Fay et al., 1997).

Other urinary biomarkers

Various drugs have been used as non-invasive biomarkers of specific cytochrome P450 activities (Guengerich et al., 1997). Examples include coumarin for CYP2A6, debrisoquine for CYP2D6, caffeine for CYP1A2 and chloroxazone for CYP2E1. Assays for metabolites of these drugs could be useful in certain chemoprevention studies if modulation of a specific cytochrome P450 is proposed. As examples, several studies have examined the effects of watercress, a rich source of PEITC, on drug metabolism in humans. Watercress caused a decrease in levels of oxidative metabolites of acetaminophen, which was attributed to inhibition of
oxidative metabolism by CYP2E1 (Chen et al., 1996). The area under the chlorzoxazone plasma concentration–time curve was significantly increased after watercress ingestion, indicating inhibition of hydroxylation of chlorzoxazone (Leclercq et al., 1998). Watercress, however, had no effect on CYP2D6 activity as monitored with debrisoquine or CYP2A6 activity as assessed by coumarin metabolism (Caporaso et al., 1994; Hecht & Murphy, unpublished data).

Effects on nicotine metabolism may also provide support for modulation of drug-metabolizing enzymes by chemopreventive agents. Nicotine is a naturally abundant substrate in smokers who would be on these trials. In a recently completed study, we found that watercress consumption induced glucuronidation of nicotine, cotinine and 3'-hydroxycotinine, suggesting that PEITC induces UDP-glucuronosyl transferase activity in smokers (Hecht et al., 1999b).

Conclusions

Table 2 summarizes information on the potential utility of carcinogen-derived biomarkers in lung and oral cancer chemoprevention trials. The only specific adduct measurement which is practical and useful for current application is HPLC-fluorescence detection of BPDE–DNA adducts. There are some limitations to this assay. The analyte is detectable in leukocytes of a large proportion of PAH-exposed individuals only when they are GSTM1-null. Moreover, the relationship between leukocyte and lung or oral tissue levels of BPDE–DNA adducts has not been established using this assay. Urinary nitrosamino acids have already been widely applied in human trials, but these specifically relate to endogenous formation of nitrosamines, not to activation and detoxification. Measurement of urinary NNAL plus NNAL-Gluc is highly practical in smokers and can be applied in chemoprevention trials provided there is a specific a priori hypothesis. Nicotine metabolites and P450 substrates could also find potential use in specific chemoprevention trials.

Several types of biomarker are potentially applicable, but further research is necessary to improve detection methods and to test them in larger groups of individuals. Nitrosamine–DNA adducts, PAH–protein adducts, and urinary metabolites of PAH could be particularly important. Several biomarkers have already been examined in fairly extensive studies and do not appear to be suitable for chemoprevention trials because they are generally undetectable or detectable at relatively low levels; examples include O6-mG in DNA, acetaldehyde–DNA adducts and tobacco-specific nitrosamine adducts with haemoglobin. Other biomarkers may have numerous endogenous and/or exogenous sources and their relevance to cancer induction is unclear; examples are methylated haemoglobin, alkyladenines in urine and 8-oxo-dG in DNA.

Other specific carcinogen-derived biomarkers as well as less specific tests, e.g. immunoassay and 32P-postlabeling, may ultimately be applicable in chemoprevention studies. Biomarkers of the uptake and metabolism of chemopreventive agents (e.g., total isothiocyanates in urine) are becoming available and the application of these is also important for ensuring compliance and assessing individual differences in the metabolism of these agents (Chung et al., 1998).

<table>
<thead>
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<th>Table 2. Application of carcinogen-derived biomarkers in lung and oral cancer chemoprevention trials</th>
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<td>Practical for current application</td>
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<td>BPDE–DNA adducts by HPLC-fluorescence</td>
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<td>Nitrosamino acids in urine</td>
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Acknowledgements

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