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# Progress and Challenges in Selected Areas of Tobacco Carcinogenesis

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## Abstract

Tobacco use continues to be a major cause of cancer in the developed world and, despite significant progress in this country in tobacco control which is driving a decrease in cancer mortality, there are still over one billion smokers in the world. This perspective discusses some selected issues in tobacco carcinogenesis focusing on progress during the 20 years of publication of *Chemical Research in Toxicology*. The topics covered include metabolism and DNA modification by tobacco-specific nitrosamines, tobacco carcinogen biomarkers, an unidentified DNA ethylating agent in cigarette smoke, mutations in the *K-RAS* and *p53* gene in tobacco-induced lung cancer and their possible relationship to specific carcinogens, secondhand smoke and lung cancer, emerging issues in smokeless tobacco use, and a conceptual model for understanding tobacco carcinogenesis. It is hoped that a better understanding of mechanisms of tobacco-induced cancer will lead to new and useful approaches for prevention of lung cancer and other cancers caused by tobacco use.

### Keywords

tobacco specific nitrosamines; secondhand smoke; smokeless tobacco; tobacco carcinogen biomarkers

## Introduction

While the use of tobacco products continues to be an immense public health problem, and arguably the largest voluntary source of human exposure to carcinogens in the world, remarkable progress has been achieved in the past 20 years, both in our understanding of mechanisms of tobacco carcinogenesis, and in tobacco control. The clean indoor air which we now take for granted in bars, restaurants, and other public places in many countries would have been unimaginable in the mid 1980s. Public disapproval of smoking and tobacco marketing has risen to new highs. Nevertheless, there are still approximately 1.3 billion smokers in the world and hundreds of millions of smokeless tobacco users (1). Cigarette smoking causes 30% of all cancer mortality in developed countries (2), and smokeless tobacco use is an important cause of cancer, particularly in southern Asia (3,4). The goal of our research is to understand mechanisms of tobacco carcinogenesis and apply this knowledge to the prevention of tobacco-induced cancer.

I congratulate the editors of *Chemical Research in Toxicology* for establishing the premier journal in chemical aspects of toxicology, which has proven to be particularly timely for chemists interested in carcinogenesis. This perspective will cover only a few selected topics in tobacco carcinogenesis, a broad field encompassing studies of individual agents as well as

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complex mixtures, and which, if reviewed comprehensively, would probably fill this entire issue. Only certain individual agents are discussed here while others, equally important, are virtually ignored, along with inhalation studies of cigarette smoke, which have been discussed in a recent perspective (5).

## Tobacco-specific nitrosamines: metabolism, DNA adduct formation and repair

Tobacco-specific nitrosamines, first characterized with respect to their presence in tobacco products and carcinogenicity in the 1970s, have emerged clearly as one of the most important groups of carcinogens in tobacco products (3,6). Seven tobacco-specific nitrosamines have been identified in tobacco products, but two of these- 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) and *N*′-nitrosonornicotine (NNN)- are the most important because of their carcinogenic activities and their consistent presence in both unburned tobacco and its smoke, frequently in relatively considerable amounts (7). NNK selectively induces mainly lung tumors in all species tested and is particularly potent in the rat (8). NNK also causes tumors of the pancreas, nasal mucosa, and liver (8). NNN produces esophageal and nasal cavity tumors in rats and respiratory tract tumors in mice and hamsters (8). NNK and NNN are considered carcinogenic to humans by the International Agency for Research on Cancer (3).

It is well established that dialkylnitrosamines such as NNK require metabolic activation by cytochrome P450 catalyzed α-hydroxylation to exert their carcinogenic properties (9). Scheme 1 illustrates the α-hydroxylation pathways for NNK (**1**) and its major metabolite 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL, **2**), as well as the 2′-α-hydroxylation pathway for NNN (**5**) (8). α-Hydroxylation of the NNK methyl group leads to intermediate **6** which spontaneously loses formaldehyde yielding 4-(3-pyridyl)-4-oxobutanediazohydroxide (**11**). The same intermediate is formed by 2′-hydroxylation of NNN. α-Hydroxylation at the methylene group of NNK produces intermediate **7** which spontaneously decomposes to methanediazohydroxide (**12**). Similar intermediates are produced by α-hydroxylation of NNAL. Methanediazohydroxide (**12**), formed in these reactions from NNK and NNAL, reacts with DNA to produce the well known DNA adducts –, $O^6$-methyl-dGuo (**21**), 7-methyl-dGuo (**22**), and $O^4$-methyl-dThd (**23**), that are common to many methylating carcinogens. But DNA adduct formation by intermediates **11** and **13** was unknown in 1986. Subsequently, it was found that neutral thermal or acid hydrolysis of DNA from NNK, NNN or NNAL-treated animals produced 4-hydroxy-1-(3-pyridyl)-1-butanone (HPB, **20**), confirming this pathway of DNA alkylation resulting in pyridyloxobutyl (POB)-DNA adducts (8,10–12). Released HPB could be quantified by GC-MS and its presence was established in the lung DNA of smokers, as well as in NNK-treated rodents (8,13,14). The structures of the adducts formed by this pathway remained elusive until the late 1990s when Peterson and co-workers characterized $O^6$(POB-1-yl)dGuo (**14**)(15), a highly mutagenic DNA adduct (16), and 2003-4 when our group identified adducts **15**–**19** and **24**–**28** as summarized in Scheme 1 (17–19).

There is no doubt that cytochrome P450 catalyzed α-hydroxylation of NNK is critical for its carcinogenicity. A review published in 1988 mentions the importance of characterizing which P450s are involved in NNK metabolism, but no data were available at that time (6). Presently, steady state kinetic parameters for P450 catalyzed NNK metabolism have been reported for 8 human enzymes, 2 rabbit enzymes, 5 rat enzymes, and 2 mouse enzymes (20). Human P450s 2A13 and 2B6, rat P450 2A3, and mouse P450 2A5 may be the most important catalysts of NNK metabolic activation in these species, based on their kinetic parameters. The critical role of mouse P450 2A5 has been clearly shown by deuterium labeling studies which demonstrated the stereoselective abstraction of the 4(*R*)- hydrogen of NNK, leading to a cascade of events resulting in lung tumor induction in A/J mice (21)(Figure 1). Thus, 4(*R*)-[4-$^2H_1$]NNK was significantly less tumorigenic than 4(*S*)-[4-$^2H_1$]NNK due to a demonstrated deuterium isotope

effect in the metabolism of this compound by P450 2A5; levels of $O^6$-methyl-dGuo were also reduced in the lungs of mice treated with 4(*R*)-[4-$^2H_1$]NNK compared to NNK or 4(*S*)-[4-$^2H_1$]NNK (21). Earlier, we demonstrated a high correlation between persistent $O^6$-methyl-dGuo in this model and the number of lung tumors per mouse, as well as the presence of G → A transition mutations in the *k-ras* gene isolated from the lung tumors (22,23). This mutation, shown by others to rapidly cause lung cancer in mice (24), is a known consequence of miscoding due to $O^6$-methyl-dGuo (25). Collectively, these data provide powerful support for the events summarized in Figure 1 and demonstrate the critical nature of the initial P450-catalyzed hydrogen abstraction, without which tumor induction would not occur.

Evidence from deuterium labeling studies and the formation and persistence of $O^6$-methyl-dGuo and POB-DNA adducts in Clara cells and type II cells of the rat lung demonstrate that both types of adducts are likely crucial in the induction of rat lung tumors by NNK (8). These studies were also bolstered by a correlation between the inhibitory effects of 2-phenethyl isothiocyanate (PEITC) on rat lung NNK carcinogenicity and POB-DNA adduct formation in type II cells of the lung (26). PEITC exerts its protective effects mainly through the inhibition of rat lung P450s, and is also a potent inhibitor of human P450 2A13 ($K_i$ = 30 nM) (27). The formation and persistence of individual POB-DNA adducts has recently been characterized in the lung of rats treated chronically with NNK or the enantiomers of NNAL (28). $O^2$(POB-1-yl)dThd, 7(POB-1-yl)Gua, $O^2$(POB-1-yl)Cyt, and $O^6$(POB-1-yl)dGuo, listed in order of their concentrations, were detected at each time point. Adduct levels in lung were similar in rats treated with NNK or (*S*)-NNAL, and far less in rats treated with (*R*)-NNAL (Figure 2). These results are consistent with studies that showed the accumulation of (*S*)-NNAL in rat lung, possibly at a receptor site (29). The current model for the organoselective carcinogenicity of NNK in the rat lung is illustrated in Figure 3. Upon administration to the rat, NNK is extensively reduced mainly to (*S*)-NNAL which binds to an as yet unknown receptor, while (*R*)-NNAL is rapidly glucuronidated (29) and eliminated from the body. This receptor binding leads to accumulation of (*S*)-NNAL in the lung. It is gradually released and re-oxidized to NNK, which then undergoes α-hydroxylation producing persistent $O^6$-methyl-dGuo and POB-DNA adducts, resulting in mutations and lung tumor induction.

Mouse P450 2A5 is an excellent catalyst of 5′-hydroxylation of both (*R*)- and (*S*)-NNN, and several other P450 2A enzymes including rat 2A3, mouse 2A4, human 2A6, and human 2A13 are also reasonably efficient (30). Lower activity was found for 2′-hydroxylation of (*R*)-NNN, and none for 2′-hydroxylation of (*S*)-NNN. Tissue specific 2′-hydroxylation of NNN by a high affinity P450 is responsible for its carcinogenicity in the rat esophagus, but this enzyme, which is clearly not P450 2A3, has not been identified (8,31). As shown in Scheme 1, 2′-hydroxylation by the unidentified esophageal P450 leads to the same POB-DNA adducts as produced by methyl hydroxylation of NNK, and these have been recently quantified in the esophagus of rats treated chronically with (*S*)-NNN or (*R*)-NNN, with the former producing higher adduct levels (32). Adducts formed by 5′-hydroxylation of NNN have also been characterized recently (33). There is no doubt that tissue specific α-hydroxylation of NNN is responsible for its carcinogenicity in the rat esophagus.

There is considerable evidence that elements of the pathway illustrated in Figure 3 are also present in human lung. NNK is readily reduced to (*S*)-NNAL by all human tissues tested including lung (34). While specific accumulation of (*S*)-NNAL in human lung has not been reported, this enantiomer is more slowly excreted in human urine than is (*R*)-NNAL, and the elimination half-life of NNAL (40–45 days) in smokers is unusually long for a small water soluble molecule (35,36). P450 2A13 is expressed in human lung, as are other P450s which metabolize NNAL and NNK, and POB-DNA adducts have been detected in human lung, with levels higher in smokers than in non-smokers (13,20,37,38). Robust metabolism of NNK in human fetal nasal microsomes, catalyzed by P450 2A13, has also been demonstrated (39).

While the human lung metabolism and DNA binding of NNK require further characterization, the results obtained to date support parallel human and rat mechanisms.

Impressive advances have occurred in our understanding of the repair of DNA adducts of NNK. Both $O^6$-methyl-dGuo and $O^6$(POB-1-yl)dGuo are repaired by $O^6$-alkylguanine-DNA alkyltransferase (AGT) (40,41). POB-DNA adducts are repaired by AGT in a reaction that results in pyridyloxobutyl transfer to the active site cysteine, similar to $O^6$-methyl-dGuo. Human AGT variants differ in their ability to repair $O^6$-methyl-dGuo and $O^6$(POB-1-yl)dGuo. The AGT mediated repair of $O^6$(POB-1-yl)dGuo was also affected by sequence context, more so than the repair of $O^6$-methyl-dGuo. These effects undoubtedly contribute to the risk of tobacco-related cancer upon exposure to NNK and NNN.

While NNAL (**2**, Scheme 1) was first identified as a metabolite of NNK in 1980 (42), NNAL *O*-glucuronidation was not established for another 10 years (43). The extensive formation of this metabolite in the patas monkey suggested that it could be a biomarker for human exposure to NNK (44). This possibility was realized with the development of an analytical method, first reported in 1993 (45). As discussed below, urinary total NNAL has emerged as a powerful biomarker for assessing human exposure to NNK. Pyridine-*N*-glucuronides of NNAL are also formed metabolically in humans (46).

In summary, significant progress has been achieved in the past 20 years in our understanding of tobacco-specific nitrosamine metabolism, DNA interactions and repair. These studies provide definitive mechanistic insights pertinent to the organoselectivity of these compounds for induction of lung and esophageal tumors in rodents, and possibly in humans. This work has led to the development of exposure biomarkers for both NNK and NNN, and application of these has impacted tobacco control as described below. While quantitative estimates of tobacco-specific nitrosamine exposure are now available from this biomarker work, definition of the corresponding situation in DNA of human tissues, particularly the lung, remains sketchy. This is a critical area for future research involving translation of our deep mechanistic understanding in laboratory animals to the human setting. Ultimately, this could lead to a better understanding of mechanisms and susceptibility to tobacco-induced cancer, and possibly cancer in general.

## Tobacco carcinogen biomarkers

Tobacco carcinogen biomarkers are substances measurable in human body fluids or tissues. These biomarkers are specifically related to tobacco carcinogens. The “Hoffmann list” of over 60 carcinogens in cigarette smoke is considered the definitive catalogue of its major cancer causing agents and includes polycyclic aromatic hydrocarbons (PAH), nitrosamines, aromatic amines, aldehydes, volatile organic compounds, metals, and others (47,48). Examples of tobacco carcinogen biomarkers include tobacco carcinogens or their metabolites in breath, blood, or urine; tobacco carcinogen-DNA adducts; and tobacco carcinogen-protein adducts. The International Agency for Research on Cancer (IARC) monograph entitled “Tobacco Smoking”, published in 1986, did not contain any references describing tobacco carcinogen biomarkers (49). In contrast, there were over 350 citations on this topic **i**n the 2004 IARC monograph entitled “Tobacco Smoke and Involuntary Smoking”, a clear demonstration that this critical area of tobacco carcinogenesis has evolved remarkably in the past 20 years (50). Applications of tobacco carcinogen biomarkers include determining carcinogen dose in people who use tobacco products and in non-smokers exposed to secondhand smoke, identifying inter-individual differences in the uptake, metabolic activation, and detoxification of tobacco carcinogens, and ultimately predicting which tobacco user is susceptible to cancer.

Measurement of carcinogen-DNA adducts potentially can provide the most direct link between cellular exposure and cancer, as DNA adducts are critical in the carcinogenic process. But

DNA adducts are challenging to quantify because their levels are extremely low, frequently ranging from 1 per $10^6$ to 1 per $10^8$ normal bases in humans (51), and the tissue samples containing them are often available in only small quantities. In recent years, the sensitivity of mass spectrometers has improved dramatically, and the routine detection of amol levels of underivatized DNA adducts is now feasible (52). Although there are still relatively few examples of quantitation of specific DNA adducts in tissues of smokers using mass spectrometry, HPLC-fluorescence, HPLC with electrochemical detection, or postlabelling techniques, this literature is expanding rapidly and includes quantitation of DNA adducts of benzo[*a*]pyrene (BaP), tobacco-specific nitrosamines (e.g. HPB releasing adducts of NNK or NNN), alkylating agents, aldehydes and other lipid peroxidation products, and products of oxidative damage such as 8-oxo-dGuo (53–58). A much larger body of work has emerged from studies which have used the highly sensitive, but relatively non-specific $^{32}$P-postlabelling and immunoassay methods for detection of DNA adducts. The advantages and disadvantages of $^{32}$P-postlabelling and immunoassay have been discussed (59–62). In summary, major advantages include high sensitivity allowing analysis of small amounts, generally micrograms, of DNA, relative simplicity of analysis, and no requirements for expensive equipment. Disadvantages include lack of chemical specificity, particularly in $^{32}$P-postlabelling analyses, and difficulty in quantitation. Although the adducts detected using this method are often referred to in the literature as “aromatic DNA adducts”, there is strong evidence that they are not related to PAH (63). The application of these methods to tissues obtained from smokers has been extensively reviewed (61). Adduct levels are generally higher in lung tissues of smokers than non-smokers while studies using blood DNA have produced mixed results. Adducts have also been detected in many other tissues and fluids from smokers including larynx, oral and nasal mucosa, bladder, cervix, breast, pancreas, stomach, placenta, fetal tissue, cardiovascular tissues, sputum, and sperm. These studies have been comprehensively reviewed (7,61). A meta-analysis of the relationship of DNA adduct levels in smokers to cancer, as determined by $^{32}$P-postlabelling, was carried out using case control studies of lung cancer (5 studies) oral cancer (1) and bladder cancer (1). Six studies measured adducts in white blood cells and one in normal lung tissue. Current smokers showed a significant difference between cases and controls, with cases having higher adduct levels than controls (64).

Protein adduct biomarkers have also been developed. Carcinogen-hemoglobin (Hb) adducts levels have been used as surrogates for DNA adduct measurements (65,66). Serum albumin adducts could also be used in this way. Although these proteins are not considered as targets for carcinogenesis, all carcinogens that react with DNA will also react with protein to some extent. Advantages of Hb adducts as surrogates include the ready availability of relatively large amounts of Hb from blood and the relatively long lifetime of the erythrocyte in humans – 120 days – which provides an opportunity for adducts to accumulate. Studies on protein adducts in smokers have been comprehensively reviewed (7,61).

Hb adducts of aromatic amines have emerged as a highly informative type of carcinogen biomarker, with levels which are consistently higher in smokers than in non-smokers, particularly for 3-aminobiphenyl and 4-aminobiphenyl-Hb adducts. Hb binds aromatic amines efficiently because the heme accelerates the rate of nitrosoarene formation from the hydroxylamine, which is produced metabolically from the aromatic amine by P450 1A2 (67). Binding of the nitrosoarene occurs at the β-93 cysteine residue of human Hb; the adduct is hydrolyzed releasing the free amine which is quantified by GC-MS (67). Adduct levels decrease upon smoking cessation and are related to numbers of cigarettes smoked (67–69). Adducts which form with the amino terminal valine of Hb are also informative. Important examples include those derived from ethylene oxide, acrylonitrile, and acrylamide (70–72). Ethylated N-terminal valine of Hb is also higher in smokers than in non-smokers (72).

Probably the most practical biomarkers are urinary metabolites of tobacco carcinogens (73). Advantages include the ready availability of samples and the higher concentration of urinary metabolites than of most adducts. Metabolites of PAH and tobacco-specific nitrosamines, and mercapturic acids derived from benzene, acrolein, and 1,3-butadiene are among the most commonly used urinary biomarkers. Among PAH metabolites, 1-hydroxypyrene (1-HOP) has probably been used more extensively than any other biomarker. Pyrene, a non-carcinogen, is a component of all PAH mixtures, and 1-HOP, a metabolite of pyrene which can be readily measured by HPLC with fluorescence detection, has been used in many studies to assess PAH uptake, which is generally 2-3 times higher in smokers than in non-smokers (73,74). We have introduced *r*-1,*t*-2,3-*c*-4-tetrahydroxy-1,2,3,4-tetrahydrophenanthrene (PheT, **34**,Scheme 2) as a biomarker of PAH uptake *plus* metabolic activation, as the metabolic pathway from phenanthrene (**30**), a non-carcinogenic PAH, to phenanthrene diol epoxide (**33**) and ultimately to PheT is highly analogous to that involved in the metabolic activation of BaP to its diol epoxide metabolite (75,76). PheT measurements will hopefully identify those smokers who are particularly able to catalyze this deleterious pathway.

Total NNAL, the sum of NNAL and its glucuronides, has emerged as a very useful biomarker of NNK exposure (73,77,78). The tobacco-specificity of NNK, and therefore total NNAL, is a key feature of this biomarker because studies in which it is applied are not confounded by environmental or dietary exposures. Total NNAL has been used in many studies to estimate lung carcinogen uptake in smokers. In one, smokers reduced their number of cigarettes smoked per day, but there was not a corresponding decrease in NNK uptake due to smokers' compensation (79,80). In another, NNK and PAH uptake were compared in smokers of regular, light, and ultra-light cigarettes, and found not to differ, consistent with epidemiologic studies which demonstrate no protection against lung cancer in smokers of light compared to regular cigarettes (81). Other studies evaluated NNK uptake in smokers who switched from their current cigarette brand to products purported to be less hazardous, but the results generally did not support the claims of the product manufacturers (82–84). The most useful application of total NNAL has perhaps been in studies of lung carcinogen uptake by non-smokers exposed to secondhand cigarette smoke (78). The sensitivity and specificity of this biomarker are ideal for such studies, and it is the only tobacco carcinogen biomarker until now consistently elevated in non-smokers exposed to secondhand smoke. The results of these studies demonstrating NNK exposure throughout life in non-smokers exposed to secondhand smoke are summarized in Table 1 (85). These results have had a significant impact on legislation prohibiting indoor smoking because non-smokers are highly adverse to the idea of having a lung carcinogen in their urine.

*S*-Phenylmercapturic acid, formed as a result of glutathione conjugation of benzene oxide, a metabolite of the human leukemogen benzene, is a highly practical and specific biomarker of benzene uptake (86,87). Similarly, 3-hydroxypropylmercapturic acid, formed from acrolein, can be readily quantified in human urine (88). Both of these mercapturic acids occur in significantly higher amounts in smokers than in non-smokers and decrease upon smoking cessation.

In summary, urinary biomarkers of tobacco carcinogen exposure are readily measured and have been applied in many studies evaluating toxicant uptake by tobacco users. These biomarkers in particular are likely to play a major role in evaluating potential tobacco related harm in the coming era of tobacco product regulation.

## A DNA ethylating agent in cigarette smoke

Recent evidence indicates that there is a DNA ethylating agent present in cigarette smoke, which could not logically be derived from any of the compounds on the "Hoffmann list" of

tobacco carcinogens. Two reports showed increased levels of 3-ethyladenine in smokers' urine. In one study, the amount of urinary 3-ethyladenine was more than 5 times greater on days when individuals smoked than on days when they didn't (89). Urinary 3-ethyladenine was also 5–8 times higher in smokers than in non-smokers in that investigation. In a second study, urinary 3-ethyladenine increased in some smokers, on days when they smoked, and a correlation was seen between cigarettes per day and 3-ethyladenine excretion (90). 3-Ethyladenine in urine most likely results from reaction of an ethylating agent with adenine in nucleic acids followed by depurination or repair. Consistent with this, Singh and Farmer demonstrated that cigarette smoke ethylates DNA in vitro, as determined by quantitation of 7-ethyl-Gua (91). Other studies in humans are consistent with this finding as smokers have higher levels of 7-ethyl-Gua in urine (89,90,92), $O^4$-ethyl-dThd in lung (93), and ethylvaline in Hb than do non-smokers (72). The origin of this DNA ethylating agent in cigarette smoke is unknown, but we have suggested ethyl nitrite and endogenous nitrosation of ethyl amine as two possibilities (72). This requires further research because an ethylating agent can produce DNA adducts with miscoding properties, thus potentially contributing significantly to tobacco carcinogenesis.

## Mutations in *K-RAS* and *p53* in lung cancer

The first studies demonstrating the common occurrence of mutations in the *K-RAS* oncogene and the *p53* tumor suppressor gene in lung cancer appeared during the 20 year lifetime of *Chemical Research in Toxicology*. Mutations in *K-RAS* have been found in approximately 30 to 40 percent of adenocarcinomas of the lung, but infrequently in other lung tumor types or in lung tumors from non-smokers (94–97). All mutations have been found in codons 12, 13, and 61, with mutations in codon 12 being by far the most common. Most of the codon 12 mutations are G→T transversions, GGT → TGT or GGT → GTT. A similar pattern of mutations is found in mouse lung tumors induced by PAH, providing support for their role as causes of lung cancer, although it should be noted that other tobacco smoke carcinogens can produce the same mutations (98). While it may not be possible to ascribe such changes to a particular tobacco smoke carcinogen, their detection in tobacco carcinogen induced lung tumors as well as lung tumors from smokers certainly supports the general hypothesis that these mutations result from DNA adducts of metabolically activated tobacco carcinogens.

The *p53* gene is the most commonly mutated tumor suppressor gene in lung cancer. *p53* mutations occur in about 40% of human lung cancers, and are generally more common in smokers than in non-smokers (53,99). Significantly higher occurrences of G → T transversion mutations have been observed in the *p53* gene in lung cancer in smokers than in non-smokers (53). *p53* mutations in lung cancer are observed at “hotspots” within the DNA binding domain of the p53 protein. The major lung cancer mutation hotspots are found at codons 157, 158, 245, 248, 249, and 273 (53). A remarkable concordance was observed between the occurrence of these mutational hotspots and the pattern of DNA adduct formation by PAH diol epoxides in reactions with the *p53* gene, as determined by ligation-mediated polymerase chain reaction or by mass spectrometric techniques (53,100–102). Adduct formation is enhanced by the presence of 5-methylcytosine in these reactive CpG dinucleotide sequences. Collectively, these data have been cited as strong evidence for the role of cigarette smoke PAH as causes of lung cancer. However, other smoke constituents can produce a similar spectrum of DNA damage. Notable among these is acrolein, which was recently shown to damage the *p53* gene in the same way as PAH diol epoxides (103). Acrolein is far more abundant in cigarette smoke than are PAHs, and acrolein-DNA adducts have been detected in human lung (55). However, acrolein is weakly or non-carcinogenic (104). While further research is necessary to determine the respective role of specific tobacco smoke carcinogens as causes of mutations in the *p53* tumor suppressor gene, the results obtained to date in aggregate support the concept that tobacco smoke constituents or their metabolically activated products directly damage the *p53* tumor suppressor gene leading to mutations and loss of normal cellular growth control mechanisms.

## Secondhand tobacco smoke and lung cancer

Major reviews covering the numerous epidemiologic studies carried out on secondhand cigarette smoke and cancer in the past 20 years have recently been published. The U.S. Surgeon General's Report on the Health Consequences of Involuntary Exposure to Tobacco Smoke concluded that "the evidence is sufficient to infer a causal relationship between secondhand smoke exposure and lung cancer among lifetime non-smokers" and "the pooled evidence indicates a 20 to 30 percent increase in the risk of lung cancer from secondhand smoke exposure associated with living with a smoker" (105). The IARC Monograph on Involuntary Smoking concluded that "there is sufficient evidence that involuntary smoking causes lung cancer in humans" and that "involuntary smoking is carcinogenic to humans" (106). These groups did not conclude that involuntary smoking causes breast cancer. Many other health effects of involuntary smoking were described in the Surgeon General's Report.

These conclusions were reached based on strong epidemiologic evidence bolstered by biomarker studies such as those described, and are biologically plausible because secondhand smoke contains all the same carcinogens that are present in the smoke inhaled by a smoker, but the dose is less. The data in Table 1 demonstrate that levels of total NNAL in the urine of non-smokers exposed to secondhand smoke are about 1–7% as great as those in smokers.

The biomarker and epidemiologic studies which demonstrate that secondhand smoke causes lung cancer have had a major impact on tobacco control. While smoking is considered a voluntary act, and many have little sympathy for the adverse health effects which smokers bring upon themselves, secondhand smoke is a different issue because non-smokers are being affected by the smoke of others. This non-voluntary health harm has spurred legislation in many states to protect non-smokers in workplaces, bars and restaurants. Currently, 20 states and several countries have laws prohibiting smoking in bars and restaurants, legislation that would have been considered improbable 20 years ago. Regulation of indoor smoking reduces cues for smoking, reduces the amount smoked, and ultimately will change social norms. Thus, regulation of indoor smoking has become one of the pillars of tobacco control, along with aggressive counter-advertising and taxation, shown to be effective in decreasing smoking prevalence (107). That such approaches are having an impact is demonstrated in Figure 4A, from the Centers for Disease Control NHANES study, showing a consistent decrease in median serum concentrations of the major nicotine metabolite cotinine, an accepted biomarker of tobacco smoke exposure, in U.S. non-smokers in the period 1988-2002. This is genuine progress which parallels a consistent decline in smoking prevalence in the U.S. in approximately the same period (Figure 4B).

## The re-reemergence of smokeless tobacco

In 1986, the author co-authored a paper entitled "The Reemergence of Smokeless Tobacco" which described the rise in smokeless tobacco use in the U.S., mostly as oral moist snuff, and particularly among young males (108). Smokeless tobacco, an accepted cause of oral cancer, is contaminated with ppm quantities of carcinogenic tobacco-specific nitrosamines, levels generally 1000 times greater than those of nitrosamines in any other consumer product. That year, the U.S. Congress enacted a law requiring health warning labels on packages of smokeless tobacco and a ban on electronic advertising. Sales of moist snuff have continued to increase in this country. In the past several years, a new concept has emerged. Responsible members of the tobacco control community support the idea of using "low nitrosamine" moist snuff as a substitute for cigarette smoking (109,110). The rationale for this is that moist snuff is demonstrably less carcinogenic in humans, and less toxic in other ways, than cigarette smoking, because it lacks the combustion products. This concept has been backed strongly by the Swedish Match company, manufacturer of Swedish moist snuff (known as "snus") which is

widely used by men in that country, in which the prevalence of cigarette smoking and the incidence of lung cancer among males are the lowest in Europe (111). The problem with this concept is that all commercially available moist snuff products, including snus, remain contaminated with significant levels of carcinogenic tobacco-specific nitrosamines in addition to carcinogenic aldehydes, metals, and sometimes PAH. According to IARC, smokeless tobacco is a human carcinogen, causing oral and pancreatic cancer (3).

Table 2 summarizes levels of tobacco-specific nitrosamines in the most popular American smokeless tobacco brands – Copenhagen, Skoal, and Kodiak – and in Swedish products (112). Levels of NNN and NNK in Swedish products are lower than those in brands sold in the U.S., but the total amounts are still about 1-2 ppm, approximately 1,000 times higher than levels of carcinogenic nitrosamines found in cured meats or beer, which are the consumer products most commonly contaminated with such compounds. We have recently completed a study in which we compared levels of total NNAL in the urine of 182 American smokeless tobacco users and 420 smokers (113). The results demonstrated that total NNAL levels were similar in the urine of smokeless tobacco users and smokers. Other studies demonstrated that users of Swedish snus did experience a reduction in total NNAL, compared to when they used American smokeless brands, but the levels were still significantly higher than when these subjects abstained from use with the aid of nicotine replacement therapy (84). The results of our studies do not support the concept that smokers should switch to smokeless tobacco. Advocating for the use of smokeless tobacco as a substitute for smoking may have the unintended consequence of increasing the use and sales of smokeless tobacco products which lead to similar uptake of tobacco-specific carcinogens as cigarettes. Long term use of nicotine replacement therapy may be a better option.

## A conceptual model for understanding mechanisms of tobacco carcinogenesis

Figure 5 presents our conceptual model for understanding mechanisms of tobacco induced cancer (114,115). The central track of this figure depicts the major established pathways of cancer causation by cigarette smoke. People start smoking for all the wrong reasons, usually as teen-agers. They become addicted to nicotine and cannot stop. Nicotine is not a carcinogen, but each puff of each cigarette contains a mixture of over 60 established carcinogens, many of which require metabolic activation, usually by cytochrome P450 enzymes, to be converted to electrophiles that react with DNA producing adducts (48,116). Cytochrome P450s 1A1 and 1B1, which are inducible by cigarette smoke via interactions with the Ah receptor, are particularly important in the metabolic activation of PAH, and the induction of these enzymes may be a critical aspect of cancer susceptibility in smokers (117). Cytochrome P450s 1A2, 2A6, 2A13, and 2E1 are also important in the activation of cigarette smoke carcinogens. Competing with activation is metabolic detoxification, resulting in excretion of carcinogen metabolites: GSTs and UGTs are particularly important in this respect. It is widely hypothesized that individuals with a higher activation and lower detoxification capacity are at highest risk for tobacco induced cancer. This reasonable hypothesis requires further investigation.

DNA adducts are absolutely central to the carcinogenic process, and there is considerable evidence that these are higher in smokers than in non-smokers (61). Cellular repair systems can remove DNA adducts and maintain a normal DNA structure (118). These systems include direct base repair by alkyltransferases, excision of DNA damage by base and nucleotide excision repair, mismatch repair, and double-strand break repair. If DNA adducts persist unrepaired, they can cause miscoding during replication when DNA polymerase enzymes process them incorrectly (119). There is considerable specificity between specific DNA adducts caused by tobacco smoke carcinogens and the types of observed mutations in genes

such as *K-RAS* and *p53*, as discussed above. These mutations can cause the loss of normal cellular growth control functions, ultimately resulting in cellular proliferation and cancer. Apoptosis protects the organism by removing cells with DNA damage. The balance between mechanisms leading to apoptosis and those suppressing apoptosis has a major impact on tumor growth (120).

The top and bottom tracks of Figure 5 indicate that other pathways may also contribute to tobacco carcinogenesis. Nicotine and tobacco-specific nitrosamines bind to nicotinic and other cellular receptors (121–124). This binding leads to activation of Akt, protein kinase A, and other pathways. Cigarette smoke activates the epidermal growth factor receptor and cyclooxygenase-2 (125), and causes down-regulation and loss of the Fhit tumor suppressor gene (126). Furthermore, the occurrence of cocarcinogens and tumor promoters in cigarette smoke is well established (49). Another important epigenetic pathway is the enzymatic methylation of promoter regions of genes, which can result in gene silencing or increased mutations (127). These aspects of the process have been insufficiently studied and their contribution to the overall pathway of carcinogenesis and potential importance with respect to the established central track of Figure 5 require further research.

## Conclusions

This brief perspective focused on several current topics in tobacco carcinogenesis, emphasizing progress in the past 20 years and identifying some current questions and controversies. There is no doubt that impressive progress has been made since the first issue of *Chemical Research in Toxicology* appeared – both in basic research and in tobacco control. Further, it is evident that advances in basic research have had an impact on tobacco control, partly through the development and application of tobacco carcinogen biomarkers. But research in tobacco carcinogenesis presents an ever changing and challenging landscape because an economically powerful industry constantly re-invents itself and operates on a global scale. Additional resources should be devoted to research in this area, because tobacco products cause at least 30% of all cancer mortality in the developed world. The currently declining cancer mortality rates in the U.S. are driven in part by prevention of tobacco-induced cancer, and we must do everything possible to ensure that this positive trend continues.

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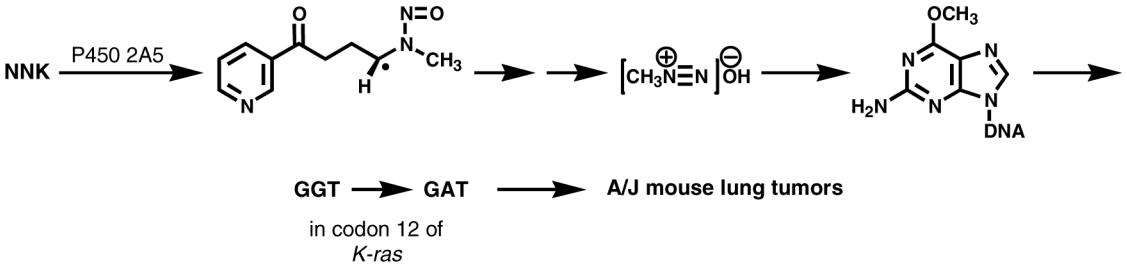



**Figure 1.**

Abstraction of the prochiral rear 4-hydrogen of NNK, catalyzed by mouse lung P450 2A5 (and possibly other P450s), is the requisite step which initiates a cascade of events leading to lung tumor formation in the A/J mouse. A single dose of 10 μmol of NNK induces about 10 lung tumors per mouse after 16 weeks in this model without the need for any exogenous tumor promoter or genetic manipulation (136). Deuterium labeling studies demonstrate that levels of $O^6$-methyl-dGuo in DNA and lung tumor multiplicity are significantly decreased in animals treated with 4(*R*)-[4-$^2H_1$]NNK compared to those treated with unlabelled or 4(*S*)-[4-$^2H_1$]NNK (21). The initially formed intermediate shown here is converted to α-methylene-hydroxyNNK (**7**,Scheme 1) which spontaneously yields methanediazohydroxide and the methyl diazonium ion (shown). The latter reacts with DNA to produce the adduct $O^6$-methyl-dGuo. Lung tumor multiplicity is highly correlated with levels of persistent $O^6$-methyl-dGuo in DNA (22). This DNA adduct causes G → A mutations in codon 12 of the *k-ras* oncogene leading to lung tumor formation (23,24).

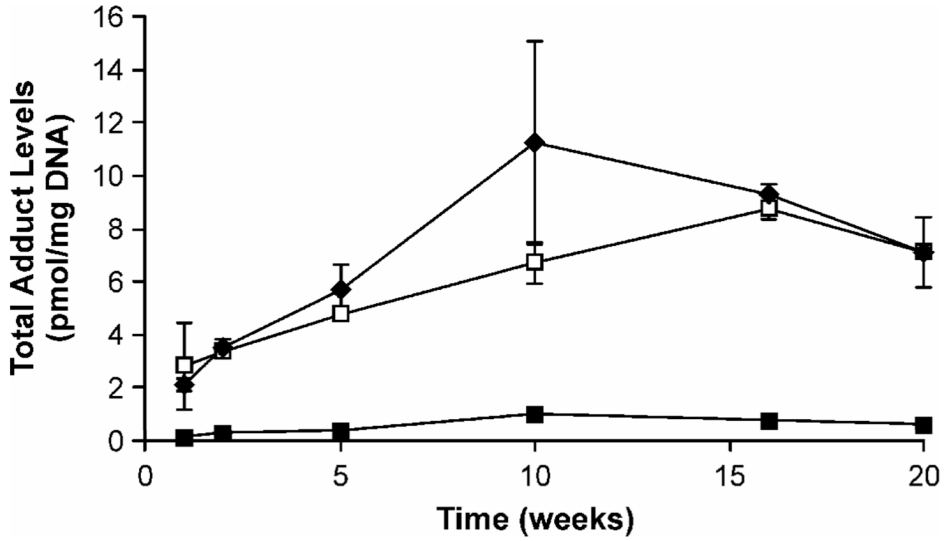



**Figure 2.**

Total POB-DNA adduct levels in the lungs of rats treated with NNK (closed diamonds), (*S*)-NNAL (open squares) or (*R*)-NNAL (closed squares). Rats were treated with 10 ppm of each compound added to their drinking water and were killed at the time intervals shown. Individual POB-DNA adducts were analyzed by LC-ESI-MS/MS-SRM as described (28).

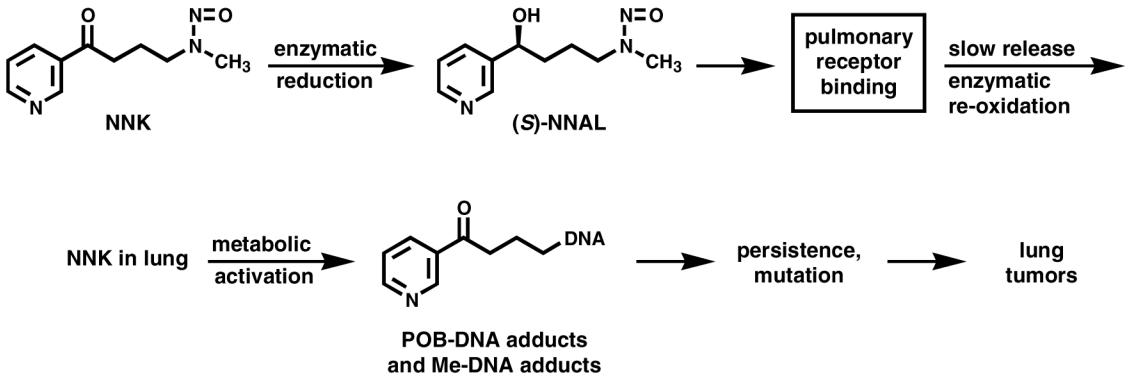



**Figure 3.**

Current model for the selective induction of induce lung tumors in rats treated with NNK. Upon administration to rats, NNK is enzymatically reduced to NNAL, with (*S*)-NNAL predominating (34). (*S*)-NNAL is hypothesized to bind to an as yet uncharacterized pulmonary receptor causing its accumulation and persistence in the lung (29). It is presumed to be slowly released from this receptor site and enzymatically reconverted to NNK, at least in part by P450s (20). This NNK then undergoes metabolic activation by P450 2A3 and other P450s in the lung, resulting in POB-DNA adducts (shown) and Me-DNA adducts (see also Scheme 1) (8,20). The persistence of these adducts in Type II cells and Clara cells of the rat lung is believed to result in mutations and lung tumor induction.

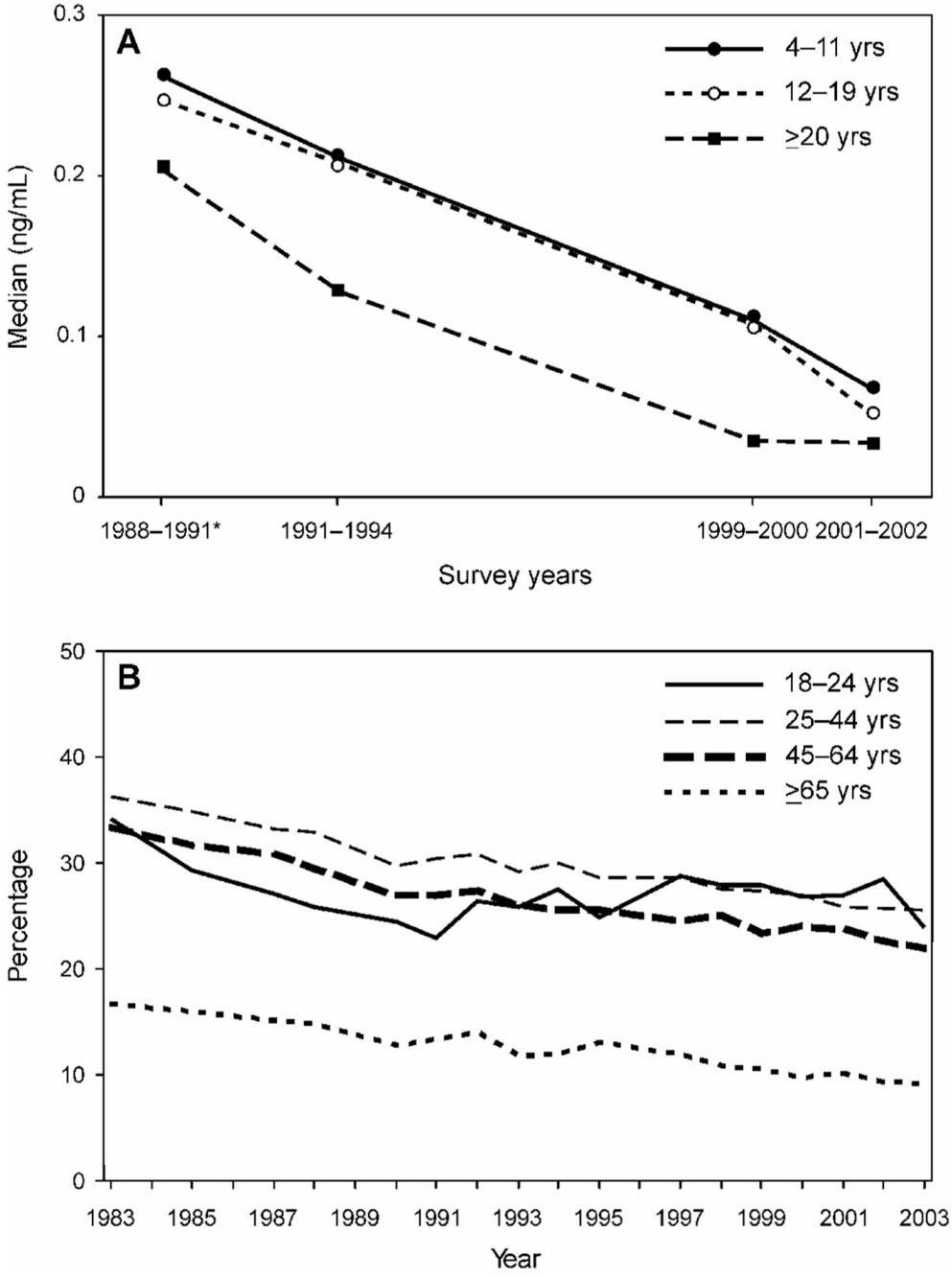



**Figure 4.**

**A.** Median serum cotinine levels in non-smokers, by age group, 1988–2002, according to data from the Centers for Disease Control and Prevention NHANES study, (137). **B.** Sustained decline of smoking prevalence in the United States, from 1983–2003 (138).

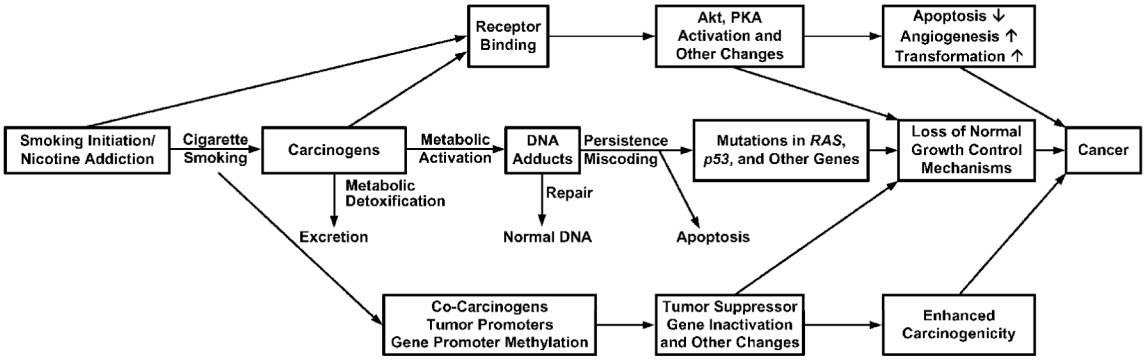
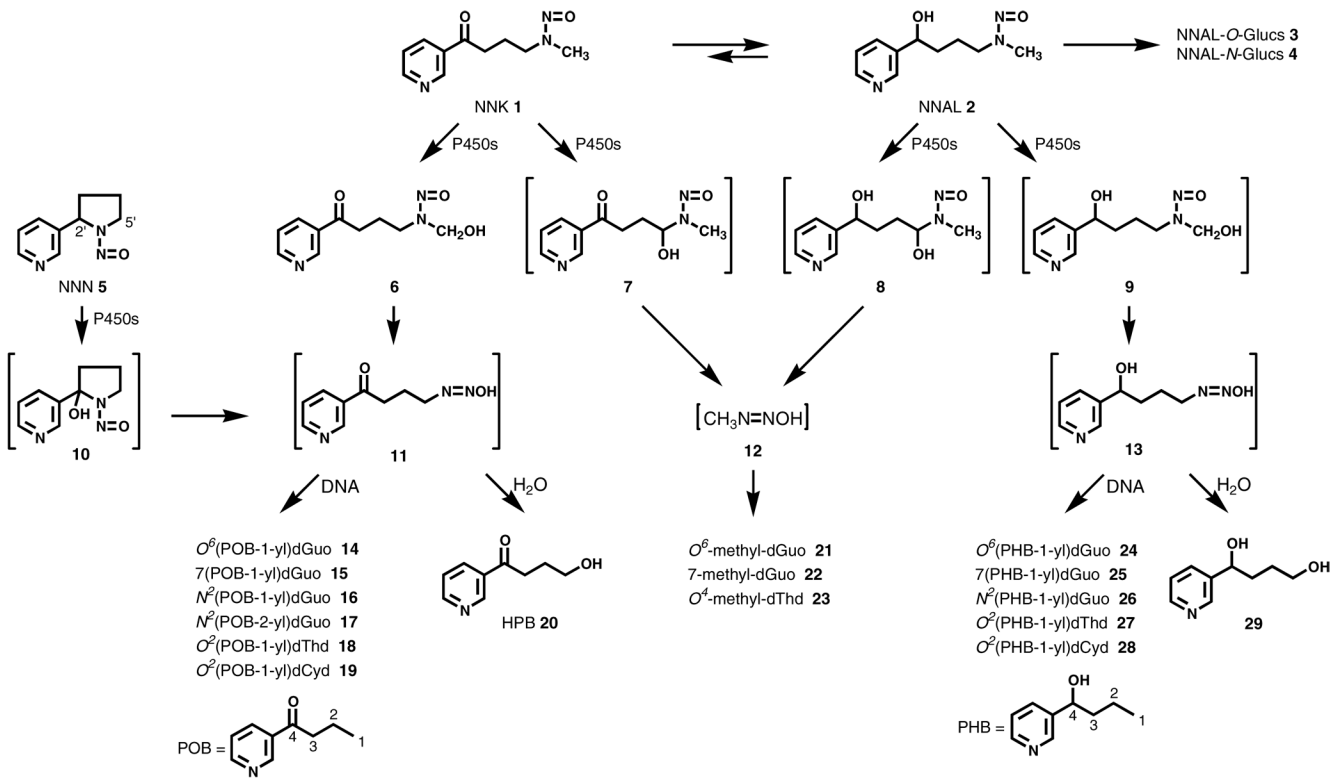
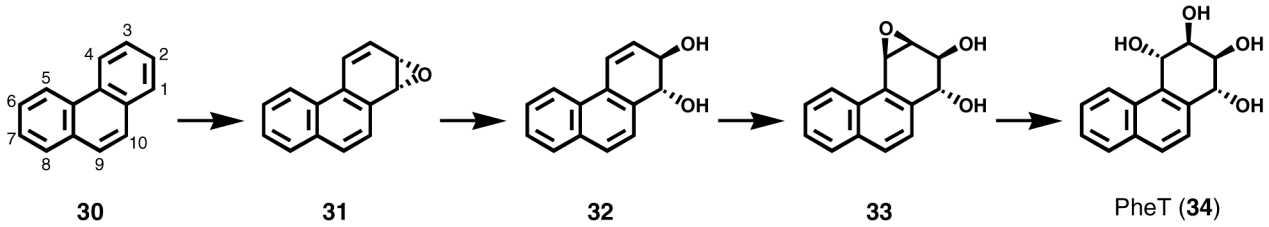



**Figure 5.**

Conceptual model for understanding mechanisms of tobacco carcinogenesis. The central track involving carcinogen-DNA adduct formation and consequent mutations in critical genes is the major accepted pathway. The top and bottom tracks also contribute but their roles are less well defined. Molecular pathways involved in the box labeled “Loss of Normal Growth Control Mechanisms” have been described by Weinberg (139).




**Scheme 1.**
DNA adduct formation from NNK, NNN, and NNAL.




**Scheme 2.**
Metabolism of phenanthrene (**30**) to a bay region diol epoxide (**33**) and PheT (**34**).

**Table 1**

Non-smokers' exposure to NNK throughout life by measurement of urinary total NNAL

| Exposed Group | Type of Exposure | Total NNAL (fmol/ mL urine) | % of Amount in Smokers' Urine[a] | Reference |
|---|---|---|---|---|
| Fetus | Transplacental | 25 ± 29 (amniotic fluid) | 1.3 | (128) |
| Newborns | Transplacental | 130 ± 150 | 6.5 | (129) |
| Infants (<1 year old) | Air | 83 ± 20 | 4.2 | (85) |
| Elementary School Children | | | | |
| Minneapolis | Air | 56 ± 76 | 2.8 | (130) |
| Moldova | Air | 90 ± 77 | 4.5 | (131) |
| Women Living with Smokers | Air | 50 ± 68 | 2.5 | (132) |
| Hospital Workers | Air | 59 ± 28 | 3.0 | (133) |
| Casino Patrons | Air | 18 ± 15 | 0.9 | (134) |
| Restaurant and Bar Workers | Air | 33 ± 34 | 1.7 | (135) |

[a] based on 2 pmol/mL total NNAL in smokers' urine

**Table 2**

Tobacco-specific nitrosamines in smokeless tobacco products[a]

| Product | µg/g product (wet weight) | | | | |
|---|---|---|---|---|---|
| | NNN[b] | NNK | NAT | NAB | Total |
| Copenhagen | | | | | |
| Snuff | 2.2 | 0.75 | 1.8 | 0.12 | 4.8 |
| Long cut | 3.9 | 1.6 | 1.9 | 0.13 | 7.5 |
| Skoal | | | | | |
| Long cut straight | 4.5 | 0.47 | 4.1 | 0.22 | 9.2 |
| Bandits | 0.9 | 0.17 | 0.24 | 0.014 | 1.3 |
| Kodiak | | | | | |
| Ice | 2.0 | 0.29 | 0.72 | 0.063 | 3.1 |
| Wintergreen | 2.2 | 0.41 | 1.8 | 0.15 | 4.5 |
| Swedish products | | | | | |
| General | 0.98 | 0.18 | 0.79 | 0.06 | 2.0 |
| Exalt | 2.3 | 0.27 | 0.98 | 0.13 | 3.7 |

[a] reference (112)

[b] Abbreviations: NNN, *N*′-nitrosonornicotine; NNK, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone; NAT, *N*′-nitrosanatabine; NAB, *N*′-nitrosoanabasine