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Invited Review

Biochemistry, Biology, and Carcinogenicity of Tobacco-Specific *N*-Nitrosamines†

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1. Introduction

Magee and Barnes demonstrated in 1956 that NDMA[1] is a powerful hepatocarcinogen in the rat (*1*). Since then, the carcinogenic activities of over 200 *N*-nitrosamines (called nitrosamines in this article) have been established in more than 30 species (*2*−*5*). Druckrey and Preussmann suggested in 1962 that nitrosamines derived from tobacco alkaloids might be present in cigarette smoke (*6*). Boyland and co-workers showed in 1964 that NNN (Figure 1) is a pulmonary carcinogen in mice and that NAB causes esophageal tumors in rats, but they could not detect these nitrosamines in cigarette smoke (*7*, *8*). Hoffmann and co-workers were the first to quantify NNN in cigarette smoke and unburned tobacco, in 1974 (*9*, *10*). Based on the classic study by Smith and Loeppky on nitrosation of tertiary amines via iminium ions (*11*), it appeared likely that other nitrosamines would be formed from nicotine (*12*, *13*). Klus and Kuhn obtained some evidence for this but were unable to identify specific products (*12*). We synthesized NNK and NNA (Figure 1), established their formation from nicotine along with NNN and a number of other products, and detected NNK in tobacco (*13*−*16*). Bioassays unexpectedly showed that NNK was a powerful pulmonary carcinogen in the rat (*17*); at that time, most unsymmetrical nitrosamines were thought to be rat esophageal carcinogens (*2*). A large number of analytical studies carried out in laboratories in many different countries conclusively demonstrate that tobacco-alkaloid-derived nitrosamines, called "tobacco-specific nitrosamines", are present in substantial quantities in both unburned tobacco and tobacco smoke (reviewed in refs *18*−*23*). The structures of these compounds and their precursors are illustrated in Figure 1. Seven tobacco-specific nitrosamines—NNN, NNK, NNAL, NAT, NAB, *iso*-NNAL, and *iso*-NNAC—have been identified in tobacco products. NNN, NNK, and NAT generally occur in greater quantities than the others, and NNN, NNK, and NNAL are clearly the most carcinogenic. Daily exposure to tobacco-specific nitrosamines is estimated as up to 20 μg in smokers and 68 μg in snuff-dippers (*22*, *23*). (The names NNK for "nicotine-derived nitrosaminoketone" and NNA for "nicotine-derived nitrosaminoaldehyde" were devised to emphasize their relationship to NNN via the common precursor nicotine; this same trivial system was later extended to NNAL, *iso*-NNAL, and *iso*-NNAC.)

The evidence that tobacco-specific nitrosamines play an important role in cancer induction by tobacco products is strong. Smoking is by far the major cause of lung cancer, a disease which was expected to kill 1.16 million people in 1996 (*19*, *24*). In the United States, where there were 48 million smokers in 1994, over 160 000 lung cancer deaths are expected in 1998 (*25*, *26*). Approximately 500 billion cigarettes were sold in the United States in 1995 (*27*). NNK and polycyclic aromatic

† This paper is dedicated to the memory of my niece, Grace Anne Ossing, Nov 5, 1993−Dec 22, 1997. Grace's brain tumor (pineal parenchymal tumor) was diagnosed at age 1. While unrelated to the compounds discussed here, her cancer dramatically illustrates the need to understand the causes of cancer and develop preventive measures.

[1]Abbreviations: AGT, O^6-alkylguanine-DNA-alkyltransferase; ALT, alanine transaminase; AMMN, (acetoxymethyl)methylnitrosamine; AST, aspartate transaminase; BaP, benzo[*a*]pyrene; BHA, 2(3)-*tert*-butyl-4-hydroxyanisole; BHT, butylated hydroxytoluene; BITC, benzyl isothiocyanate; BOP, *N*-nitrosobis(2-oxopropyl)amine; CNPB, 4-(carbethoxynitrosamino)-1-(3-pyridyl)-1-butanone; diol, 4-(3-pyridyl)butane-1,4-diol; DMBA, 7,12-dimethylbenz[*a*]anthracene; EGCG, (−)-epigallocatechin gallate; EROD, ethoxyresorufin-*O*-dealkylase; FMO, flavin-containing monoxygenases; HPB or keto alcohol, 4-hydroxy-1-(3-pyridyl)-1-butanone; HPV, human papilloma virus; HSV-1, herpes simplex virus-1; α-hydroxymethylNNK, 4-(hydroxymethylnitrosamino)-1-(3-pyridyl)-1-butanone; hydroxy acid, 4-(3-pyridyl)-4-hydroxybutanoic acid; *iso*-NNAL, 4-(methylnitrosamino)-4-(3-pyridyl)-1-butanol; *iso*-NNAC, 4-(methylnitrosamino)-4-(3-pyridyl)butanoic acid; ITC, isothiocyanate; keto acid, 4-(3-pyridyl)-4-oxobutanoic acid; keto aldehyde, 4-(3-pyridyl)-4-oxobutanal; lactol, 5-(3-pyridyl)-2-hydroxytetrahydrofuran; lactone, 2-(3-pyridyl)butyrolactone; LDH, lactate dehydrogenase; 7-mG, 7-methylguanine; MC, 3-methylcholanthrene; [*methyl*-D_2]NNK, [4,4-dideuterio]-4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone; [*methyl*-D_3]NNK, [*methyl*-D_3]-4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone; NAB, *N*′-nitrosoanabasine; NNAL, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol; NAT, *N*′-nitrosoanatabine; NDMA, *N*-nitrosodimethylamine; NNA, 4-(methylnitrosamino)-4-(3-pyridyl)butanal; NNAL(ADP)$^+$, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol adenosine dinucleotide phosphate; NNAL-Gluc, [4-(methylnitrosamino)-1-(3-pyridyl)but-1-yl]-β-*O*-D-glucosiduronic acid; NNAL *N*-oxide, 4-(methylnitrosamino)-1-(3-pyridyl *N*-oxide)-1-butanol; NNK, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone; NNK(ADP)$^+$, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone adenosine dinucleotide phosphate; NNK *N*-oxide, 4-(methylnitrosamino)-1-(3-pyridyl *N*-oxide)-1-butanone; NNN, *N*′-nitrosonornicotine; NNN *N*-oxide, *N*′-nitrosonornicotine 1-*N*-oxide; NNKOAc, 4-(acetoxymethylnitrosamino)-1-(3-pyridyl)-1-butanone; NQO, 4-nitroquinoline *N*-oxide; O^6-mdG, O^6-methyldeoxyguanosine; O^6-mG, O^6-methylguanine; O^4-mT, O^4-methylthymidine; 8-oxo-dG, 8-oxodeoxyguanosine; PB, phenobarbital; PEITC, 2-phenylethyl isothiocyanate; PHITC, 6-phenylhexyl isothiocyanate; PROD, pentoxyresorufin-*O*-dealkylase; *p*-XSC, 1,4-phenylenebis(methylene)selenocyanate; pyridyl-THF, 2-(3-pyridyl)-tetrahydrofuran; SSB, single-strand break; TCDD, 2,3,7,8-tetrachloro-*p*-dioxin; TPA, 12-*O*-tetradecanoylphorbol 13-acetate; UDPGT, uridine 5′-diphosphoglucuronyltransferase.

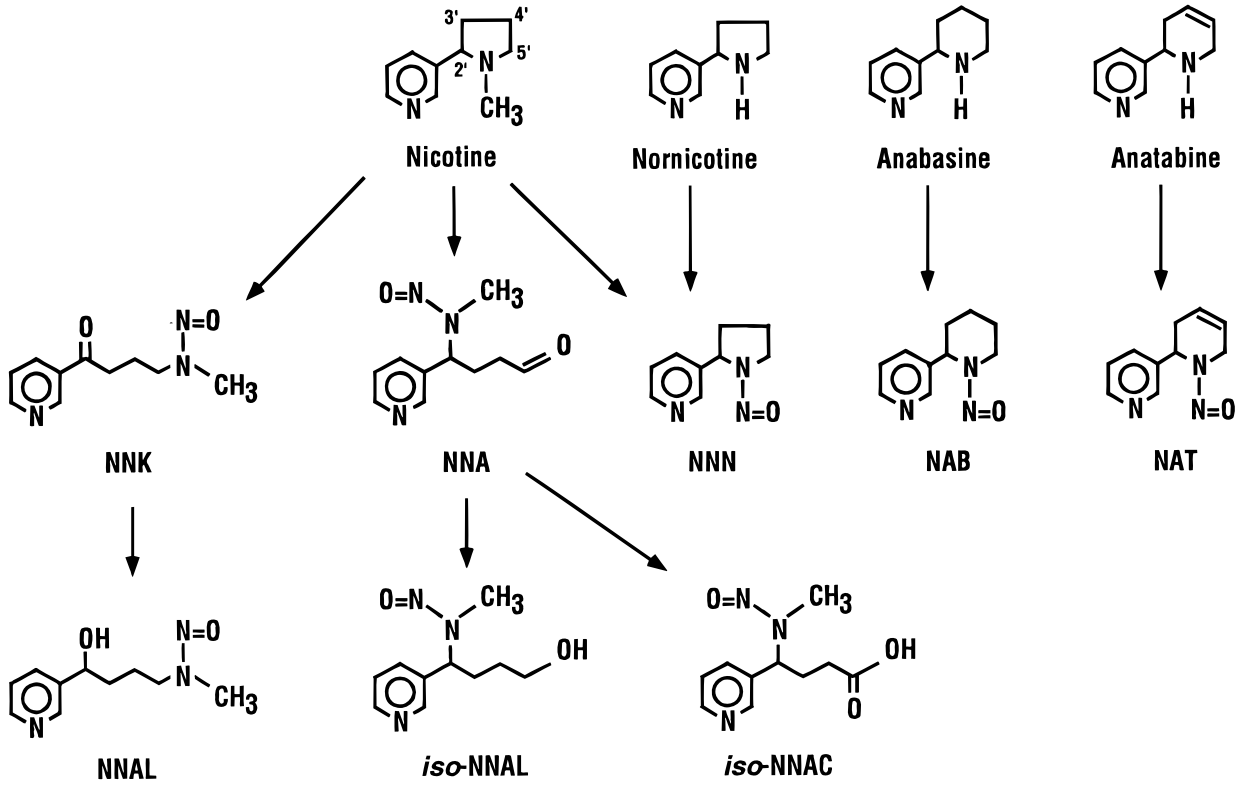

Figure 1. Structures of tobacco-specific nitrosamines and tobacco alkaloid precursors. With the exception of NNA, all have been detected in tobacco products.

hydrocarbons are the most prevalent pulmonary carcinogens in tobacco smoke; their respective roles in lung cancer induction are discussed in section 5 of this review. It is noteworthy however that NNK is a potent and selective inducer of adenocarcinoma of the lung in rodents, and the total doses required are not dissimilar from those experienced by smokers (see section 2.6). Adenocarcinoma is now the leading lung cancer type in the United States, having surpassed squamous cell carcinoma, and a recent study demonstrates that this change in histology is due to the changing cigarette, and not to improved methods of diagnosis (*28*). Smoking is an important cause of esophageal and pancreatic cancer, being responsible for 70−80% of esophageal cancer death and 25% of pancreatic cancer death in the United States (*19, 29, 30*). NNN occurs in greater concentrations in cigarette smoke than any other esophageal carcinogen (*31, 32*). NNK and its major metabolite NNAL are the only known pancreatic carcinogens in cigarette smoke (*31*−*33*). While the complexity of tobacco smoke precludes definitive assignment of cause and effect to any particular carcinogen, NNK, NNAL, and NNN certainly play a significant role in cancer induction. Unburned tobacco presents a different picture. Here, tobacco-specific nitrosamines are by far the most prevalent strong carcinogens, occurring routinely in the 1−10 ppm range in moist snuff products sold in the United States and as high as the milligram/gram range in tobacco products in other parts of the world (*18, 22, 23, 34, 35*). It is now widely accepted that NNN and NNK play a major role in oral cancer induction by smokeless tobacco, although aspects of the mechanism remain unclear (*20, 21, 32, 36*−*39*).

Tobacco-specific nitrosamines require metabolic activation to exert their carcinogenic effects. NNK, NNAL, and NNN metabolism, adduct formation, and detoxification are now quite well-understood, as presented in this review. Many of the same reactions occur in rodents and humans, although there are quantitative differences. NNK− and NNN−DNA and hemoglobin adducts, as well as urinary metabolites of NNK, have been quantified in humans. Thus, the overall pathway leading to the initiation of cancer—carcinogen exposure and uptake, metabolic activation, and DNA adduct formation—has been demonstrated for tobacco-specific nitrosamines in humans, and these data, taken together with the overwhelming epidemiologic evidence that tobacco products cause cancer in humans, directly point to the relevance of these compounds in human cancer etiology. Research on tobacco-specific nitrosamines continues to provide new insights on mechanisms of cancer induction by tobacco products.

Several previous reviews have discussed aspects of tobacco-specific nitrosamine occurrence, metabolism, carcinogenicity, and relevance to human cancer (*20*−*23, 32, 40*−*43*). There have been no comprehensive reviews of the biochemistry, biology, and carcinogenicity of tobacco-specific nitrosamines since the 1980s. The emergence of NNK as a leading model compound for studies of lung cancer mechanisms and chemoprevention of lung cancer has spurred research in this area. Considerable interest in NNN and NNK was also sparked by the reappearance of smokeless tobacco as a popular consumer product in the United States: 121 million pounds—60 million as moist snuff—was consumed in 1997 (*44, 45*). This review will attempt to cover all peer-reviewed studies on tobacco-specific nitrosamine biochemistry, biology, and carcinogenicity.

2. NNK

2.1. NNK and NNAL Metabolism. NNK metabolism pathways are illustrated in Figure 2. Five types of

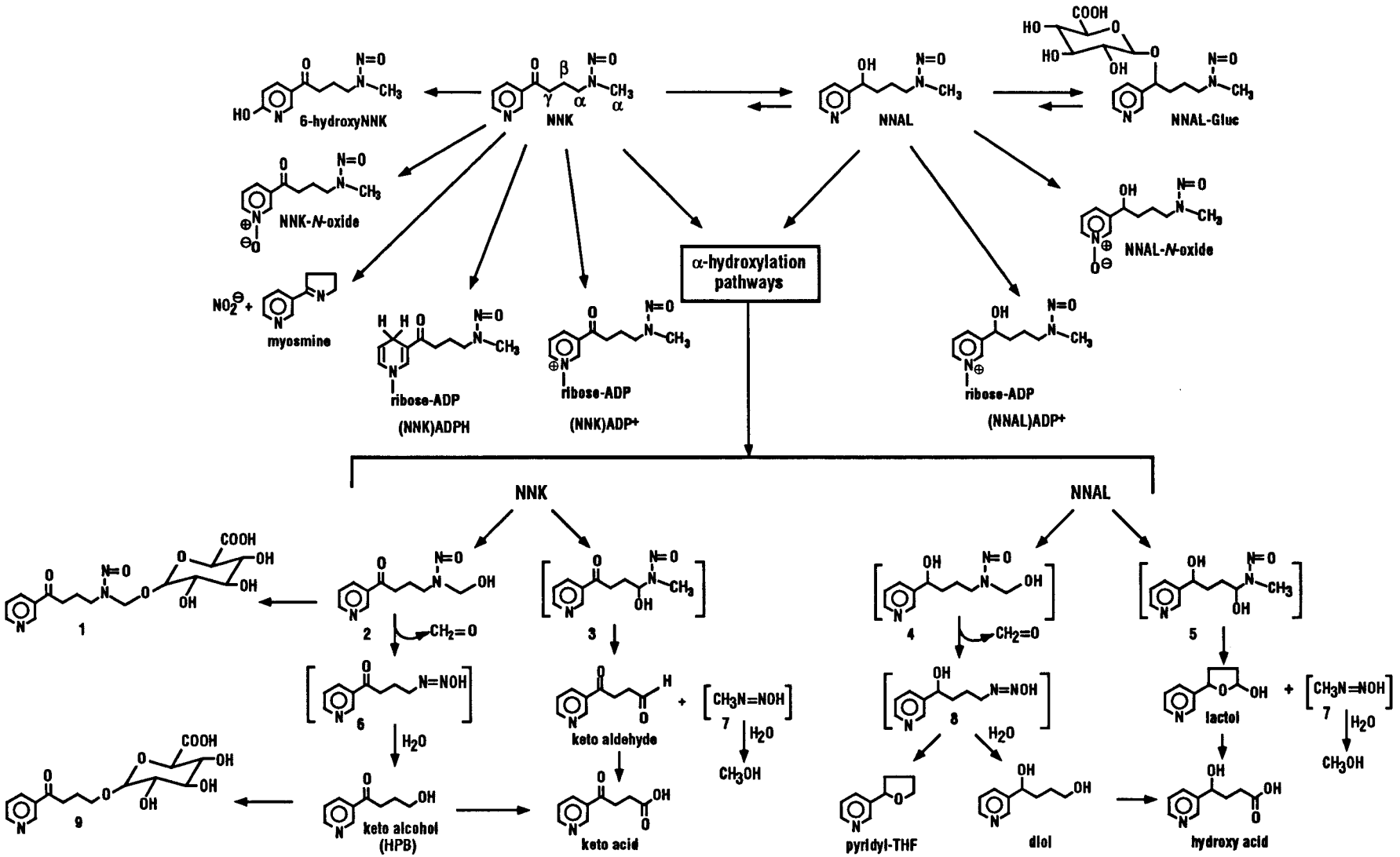

Figure 2. NNK metabolism pathways as determined by studies in laboratory animals and humans. In the ADP adducts, the pyridine nitrogen is bound to the 1′-carbon of the ribose ring. For P450 involvement, see Tables 3 and 4.

Table 1. In Vitro Metabolism of NNK and NNAL[a]

Species	Tissue	Conditions	Reference
Rat	Liver	Microsomes, tissue slices, hepatocytes	*46−68*
	Lung	Microsomes, isolated lung cells, tissue culture	*50, 58, 59, 65, 67−69, 70−73*
	Nasal mucosa	Microsomes, tissue culture	*71, 72, 74−77*
	Oral tissue	Tissue culture	*78−80*
	Esophagus	Tissue culture	*78*
	Intestine	Perfused tissue	*81*
	Pancreas	Microsomes	*62*
Hamster	Various	Tissue culture	*82*
	Liver	Microsomes	*61, 63, 68, 82−90*
	Lung, trachea	Tissue culture, cultured cells, microsomes	*68, 90, 91−95*
	Intestine	Perfused tissue	*96*
Mouse	Liver, lung, nasal mucosa	Tissue culture	*97*
	Liver	Microsomes	*98−105*
	Lung	Tissue culture, microsomes	*98−113*
	Stomach, intestine	Tissue culture, perfused tissue	*96, 109, 114*
Rabbit	Nasal mucosa	Microsomes	*76*
Pig	Lung, liver	Microsomes	*68*
Monkey	Lung, liver	Microsomes	*115*
Human	Various	Tissue culture	*116*
	Liver	Microsomes	*117, 118*
	Lung	Microsomes	*117, 119*
	Oral tissue	Tissue or cell culture	*120*
	Red blood cells	Cell culture	*60*
	Placenta	Microsomes	*121*
	Urine	Bacteria	*122*
	Pancreas	Microsomes	*123*

[a] Condensed version; full table appears in Supporting Information.

Table 2. Summary of Extents of NNK Metabolite Formation in Unpretreated Rodents and in Humans[a]

Species	Tissue	Carbonyl Reduction	Pyridine N-Oxidation	α-Methylene Hydroxylation	α-Methyl Hydroxylation	ADP Adduct
Rat	Liver	+++	+	++	++	+
	Lung	++	+++	++	++	++
	Nasal mucosa	−	+	+++	+++	NA
	Oral tissue	+++	++	++[b]	++[b]	NA
	Pancreas	++	−	−	+	++
	Intestine	+++	++	+[b]	+[b]	NA
Hamster	Liver	+++	++	+	++	NA
	Lung	++	++	+	+++	NA
	Intestine	++	+++	++[b]	++[b]	NA
Mouse	Liver	+++	−	++	++	NA
	Lung	++	+++	++	++	NA
	Intestine	+	++	+++[b]	+++[b]	NA
Human	Liver	+++	+	++	++	NA
	Lung	+++	−	+	+	NA

[a] Symbols refer to comparisons within a given tissue: +++, usually the predominant pathway; ++, one of the major pathways; +, minor pathway; −, not generally observed; NA, not analyzed. [b] Total α-hydroxylation.

transformations have been observed: carbonyl reduction, pyridine oxidation, α-hydroxylation (hydroxylation of the carbons adjacent to the *N*-nitroso group), denitrosation, and ADP adduct formation. There is presently no evidence for β- or γ-hydroxylation. Each transformation will be considered separately in the following sections on in vitro metabolism, enzyme involvement, and in vivo metabolism.

2.1.1. In Vitro Metabolism of NNK and NNAL. Studies examining the in vitro metabolism of NNK are summarized in Table 1 (*46−124*). The condensed versions of Tables 1, 5−7, 9, 10, 13, and 16−20 are presented in this manuscript. The full tables are available as Supporting Information on the ACS web site http://pubs.acs.org or in reprints. Investigations have been carried out using subcellular fractions, cell culture, tissue culture, and isolated perfused tissues from the rat, hamster, mouse, rabbit, monkey, pig, and human. Each of the five metabolic pathways will be discussed.

2.1.1.1. Carbonyl Reduction. Reduction of the NNK carbonyl group produces NNAL. Although this reaction was first documented in 1980, the absolute configuration of metabolically formed NNAL has been examined only recently (*46*, *124*). Rat liver microsomes produce mainly (*R*)-NNAL, and there appears to be considerable stereoselectivity depending on the system involved.

In many tissues, NNAL is the predominant NNK metabolite formed in vitro (Table 2). This is the case in rodent and human liver, as well as in human lung (*46*, *57*, *63*, *66*, *96*, *116−119*), and in rat intestine, but not in hamster or mouse intestine (*96*). Enzymatic activity for NNK carbonyl reduction is widely distributed. For example, human red blood cells produce substantial amounts of NNAL upon incubation with NNK (*60*). Rat and human pancreas, which have low α-hydroxylation activity, convert NNK to NNAL (*62*, *123*, *125*). An exception is the rodent nasal mucosa in which minimal NNAL formation is observed (*74*, *75*). The enzymology

of NNAL formation is discussed further in section 2.1.2.1. In many studies, P450 inducers or inhibitors modify the oxidative metabolism of NNK but have little effect on the carbonyl reduction pathway (*57*, *58*, *61*, *63*, *66*, *67*, *70*, *72*, *80*, *86*, *92*, *95*, *98*, *102*). Thus, P450s appear to play little role in NNAL formation.

NNAL is not a detoxified metabolite of NNK, since its carcinogenic activity is similar to that of NNK (*106*, *126*, *127*). This is discussed further in section 2.6. NNAL is metabolically transformed in ways similar to that of NNK, with a notable exception being formation of NNAL-Gluc. Rat hepatocytes produce mainly (*S*)-NNAL-Gluc from NNK, while fortified rat liver microsomes give mainly (*S*)-NNAL-Gluc when incubated with racemic NNAL (*64*, *67*, *124*). NNAL-Gluc is probably a detoxification product. There is no evidence that NNAL is a substrate for sulfotransferase. The oxidation of NNAL to NNK has received limited attention. It generally does not occur as extensively as reduction of NNK to NNAL (*62*, *67*). This reaction may be important because the carcinogenic effects of NNAL could be due in part to reconversion to NNK.

2.1.1.2. Pyridine Oxidation. Only oxidation of the pyridine nitrogen has been reported in vitro (Table 1). There are wide variations in the production of NNK *N*-oxide and NNAL *N*-oxide depending on species and tissue (Table 2). NNK *N*-oxide is generally the major metabolite of NNK in rat and mouse lung microsomes (*70*, *72*, *99*, *108*). In contrast, pyridine N-oxidation is a minor pathway, or not detected, in liver microsomes from non-pretreated rats and mice and in rat nasal mucosa microsomes (Table 2) (*57*, *72*, *74*, *99*). Pyridine N-oxidation is strongly induced by pretreatment with PB, but not MC in rat liver, suggesting the involvement of P450 2B1 (*57*, *66*). However, this enzyme does not appear to be involved in pulmonary *N*-oxide formation (*72*). Nevertheless, a large number of studies consistently indicate that pulmonary P450s are the major catalysts of pyridine N-oxidation as well as α-hydroxylation of NNK and NNAL. This is discussed further in section 2.1.2.2. Extensive formation of NNK *N*-oxide in rat esophagus may explain the low esophageal carcinogenicity of NNK, since NNK *N*-oxide is less carcinogenic than NNK (*78*). Pyridine N-oxidations of NNK and NNAL are observed in human liver microsomes but not in human lung (*118*, *119*).

2.1.1.3. α-Methylene Hydroxylation. Hydroxylation of the methylene carbon adjacent to the *N*-nitroso group produces the unstable intermediate α-methylenehydroxy-NNK (**3**), which spontaneously decomposes to methane diazohydroxide (**7**) (or the corresponding diazonium ion) and keto aldehyde (Figure 2). In microsomal systems, keto aldehyde is quantified as its bisulfite adduct (*99*). In the absence of bisulfite, further oxidation to keto acid is observed, and there may be other uncharacterized reactions such as protein binding. Since bisulfite can inhibit metabolism under some conditions, care must be taken in choosing the appropriate concentration (*83*, *99*, *118*, *128*).

Methane diazohydroxide (**7**) methylates DNA forming a variety of adducts, among which 7-mG and O^6-mG are commonly detected. The latter plays a significant role in NNK carcinogenicity, and consequently α-methylene hydroxylation is a metabolic activation pathway. DNA adduct formation and its significance are discussed in section 2.2. Quantitation of DNA adduct formation with added calf thymus DNA is used in some in vitro studies to assess α-hydroxylation (*50*, *71*).

α-Methylene hydroxylation of NNAL similarly gives methane diazohydroxide (**7**) and a hydroxy aldehyde which cyclizes to lactol (Figure 2). Lactol can be further oxidized to hydroxy acid. Formation of keto aldehyde from NNK and lactol from NNAL is highly correlated in human liver microsomes, indicating the involvement of the same enzyme (*118*).

α-Methylene hydroxylation of NNK is commonly observed in vitro (Table 2). It is the predominant pathway of NNK metabolism in rat nasal mucosa, along with α-methyl hydroxylation (*71*, *72*, *74*−*76*). Nasal mucosa generally has higher NNK α-hydroxylation activity than do other tissues (*47*, *82*). There is convincing evidence that multiple P450s are involved in the two α-hydroxylation pathways.

α-Methylene hydroxylation is inhibited by numerous P450 inhibitors. Interestingly, in rat lung, but not liver microsomes, it is also inhibited by chronic treatment with NNK itself, possibly due to P450 inactivation by keto aldehyde binding. Inhibition of α-methyl hydroxylation is not observed under these conditions (*67*).

2.1.1.4. α-Methyl Hydroxylation. Hydroxylation of the NNK methyl group yields α-hydroxymethylNNK (**2**; Figure 2). The formation of this metabolite has been confirmed by characterization of its glucuronide as a product of NNK metabolism in rat hepatocytes and urine (*64*). α-HydroxymethylNNK (**2**) spontaneously decomposes to formaldehyde and 4-(3-pyridyl)-4-oxobutane 1-diazohydroxide (**6**) (or the corresponding diazonium ion). The latter pyridyloxobutylates DNA and is important in NNK carcinogenesis. Reaction of diazohydroxide **6** with H_2O gives keto alcohol, also referred to as HPB (Figure 2). In some in vitro systems, such as cultured rat oral tissue or esophagus, keto alcohol is partially oxidized to keto acid (*78*). Another product which may result from α-methyl hydroxylation of NNK is myosmine; alternatively, myosmine could be produced by denitrosation of NNK (*46*, *54*).

α-Methyl hydroxylation of NNAL produces a similar cascade of products, the end results of which, in microsomal incubations, are pyridyl-THF and diol.

α-Methyl hydroxylation of NNK is assayed directly by analysis of keto alcohol (HPB) or by pyridyloxobutylation of added DNA. α-Methyl hydroxylation of NNAL is assayed by formation of diol and pyridyl-THF. The frequent use of NNK as a model compound for lung tumor induction in studies of potential chemopreventive agents has spurred a large number of studies on inhibition of NNK α-methyl and α-methylene hydroxylation by a variety of agents such as arylalkyl isothiocyanates, diallyl sulfide, aromatic aldehydes, polyphenols, nonsteroidal antiinflammatory agents, tea, capsaicin, and others (Table 1).

α-Methyl hydroxylation of NNK occurs to varying extents in in vitro studies (Table 2). It is the predominant pathway, along with α-methylene hydroxylation, in nasal mucosa microsomes and cultures. It is also a consistently major pathway in rodent lung, generally exceeding α-methylene hydroxylation (*67*, *72*, *90*, *99*, *108*). In the rat, on a per milligram of protein basis, total α-hydroxylation is greatest in the nasal mucosa followed by lung and liver (*57*, *67*, *71*, *72*, *74*, *75*). The relatively high activity in the rat lung, coupled with persistence of the resulting DNA adducts, is one reason for the sensitiv-

ity of the rat lung to NNK carcinogenesis. Nasal mucosa and liver are also targets for NNK carcinogenesis. The two α-hydroxylation pathways of NNK and NNAL occur at approximately equal rates in human liver (*118*).

2.1.1.5. Denitrosation. Only one study has examined denitrosation of NNK, a pathway that has been extensively characterized for NDMA (*54, 129*). By analogy to NDMA, denitrosation of NNK would proceed by formation of an α-carbon radical followed by elimination of NO•. The imines resulting from these reactions would hydrolyze to keto aldehyde and myosmine. Neither of these has been characterized specifically as a product of denitrosation, but enzymatic formation of nitrite from NNK by rat liver microsomes was observed.

2.1.1.6. ADP Adduct Formation. ADP adducts of NNK and NNAL—NNK(ADPH), $NNK(ADP)^+$, and $NNAL-(ADP)^+$—were characterized relatively recently, in studies of NNK metabolism with rat pancreatic or liver microsomes (*62*). $NNK(ADP)^+$ elutes close to keto acid on HPLC and could be mistaken for this metabolite. The ADP adducts have been observed only in in vitro studies to date. $NNK(ADP)^+$ and $NNAL(ADP)^+$ are formed with catalysis by NAD glycohydrolase, which also catalyzes similar reactions of nicotine, cotinine, 3-acetylpyridine, and others (*62*). The physiological role of these enzymes is not clear, nor is the potential role of the ADP adducts in NNK or NNAL carcinogenesis.

2.1.2. Enzyme Involvement in NNK Metabolism. Most studies have focused on oxidative metabolism of NNK. All evidence indicates that these reactions are catalyzed by P450s, with little, if any, involvement of other enzyme systems such as FMO (*68*). A possible exception is lipoxygenase in lung (*119*). P450 involvement in NNK metabolism is summarized in Tables 3 and 4. P450s 1A2, 2A, 2B1, and 3A play a role in oxidative metabolism of NNK in rats and mice. P450 2E1 is minimally involved in NNK metabolism in rats and mice. There appears to be considerable unidentified P450 activity. For example, NNK *N*-oxide is generally the major metabolite formed in rat and mouse lung, and there is strong evidence that this is mainly a P450-mediated reaction. However, antibody studies do not show any inhibition of this reaction. Antibodies to P450 2A1 inhibit α-hydroxylation reactions in rat and mouse lung, but this enzyme is not present in lung, suggesting a role for a related P450 2A. In addition, the effects of isothiocyanates on activities associated with P450 1A and 2B (EROD and PROD) in rat and mouse lung do not correlate with their effects on NNK metabolism although antibody inhibition studies indicate a role for these P450s (*58, 72*). Much less is known about the involvement of specific P450 forms in NNK metabolism in the hamster, although hamster liver microsomes metabolize NNK more effectively than those from rats (*63*). P450s 2B1 and 2E1 may be involved in hamster hepatic microsomal NNK metabolism, but further studies are required (*83*).

Studies on the role of human P450s in NNK metabolism are summarized in Tables 3 and 4 and described further below. Overall, it appears that P450s 1A2, 2A6, and 3A4 are most important in the hepatic metabolic activation of NNK. P450 2D6 has been studied extensively because of its possible involvement in lung cancer. It does catalyze the α-hydroxylation of NNK, but its activity is low (*117, 132, 135*).

2.1.2.1. Carbonyl Reduction. P450s are not the major catalysts of NNAL formation. Rather, this reaction is mediated by carbonyl reductases. 11-β-Hydroxysteroid dehydrogenase (EC 1.1.1.146), a microsomal enzyme responsible for the interconversion of active 11-hydroxyglucocorticoids to inactive 11-oxo forms, has been identified as one carbonyl reductase involved in the reduction of NNK to NNAL. Whether this is the major enzyme responsible for NNK reduction in mammals is not known (*141*). It has been suggested that differences in tissue expression of this enzyme, as well as genetic polymorphisms, may influence NNK carcinogenicity by affecting detoxification via NNAL-Gluc (*142*).

2.1.2.2. Pyridine Oxidation. P450 2B1 isolated from rat liver metabolizes NNK to NNK *N*-oxide in a reconstituted system (*130*). Similarly, human kidney cells transfected with P450 2B1 cDNA convert NNK to NNK *N*-oxide (*131*). Pretreatment of rats with PB induces hepatic NNK *N*-oxide formation, and treatment with PEITC results in initial inhibition, then induction of P450 2B1 activity, a pattern parallel to that observed for formation of NNK *N*-oxide from NNK in these rats (*57, 58, 66*). Collectively, there is strong evidence that P450 2B1 is one of the major rat hepatic P450 forms responsible for conversion of NNK to NNK *N*-oxide.

In rat lung, where NNK *N*-oxide is consistently the major metabolite, the picture is less clear. Although P450s appear to be the major enzymes involved in the formation of this metabolite, and there is some induction by PB treatment, studies with antibodies to P450 2B1 show no inhibition of NNK *N*-oxide formation. (*57, 72*). Moreover, there is little correspondence between the effects of PEITC on PROD activity and NNK *N*-oxide formation in rat lung. Similarly, in A/J mouse lung, antibodies to rat hepatic P450s do not inhibit NNK *N*-oxide formation, and there is no correlation between the effects of isothiocyanates on EROD/PROD activities and NNK conversion to NNK *N*-oxide (*59, 102*).

Limited data are available on the role of P450s in pyridine N-oxidation in human tissues. One study shows a correlation between formation of *N*-oxides of NNK and NNAL and P450 3A4 activity in human liver microsomes (*118*).

2.1.2.3. α-Methylene Hydroxylation. In a reconstituted system, keto aldehyde is one of the minor products of NNK metabolism by rat hepatic P450 2B1 (*130*). Antibody inhibition studies show some involvement of P450s 1A2 and 3A in rat liver α-methylene hydroxylation, and studies with inducers and inhibitors are generally consistent with a role for these two P450s as well as P450 2B1 (*57, 58*).

Antibody inhibition studies also support a role for P450 2B1 in rat pulmonary α-methylene hydroxylation (*50, 71, 72*). Antibodies to rat P450s 2A1 and 1A2 have significant inhibitory activity against keto aldehyde formation, but it is not clear which related forms are present in the lung (*72*). Formation of keto aldehyde does not correlate with PEITC effects on EROD and PROD (*58*).

Antibody inhibition studies support the involvement of P450s 1A2 and 2A1 in α-methylene hydroxylation of NNK in rat nasal mucosa, while in mouse lung they indicate a role for P450s 2B1 and 2A1 (*72*). However, P450 2A1 has not been detected in mouse lung although there may be a related form present (*102*). Inhibition is also seen with an antibody to P450 2F ($P450_{MP}$). Effects of isothiocyanates on PROD activity do not correlate with their effects on NNK metabolism to keto aldehyde in mouse lung (*59*).

Table 3. Cytochrome P450 Involvement in NNK Metabolism

Entry No.	Species	Tissue	Conditions	Main P450 Involvement	No Involvement	Reference
1.	Rat	Lung	Antibody inhibition; chemical induction/ inhibition, 2 mM NNK	2B1 > 1A1	4B1	*50*
2.	Rat	Liver	Reconstituted system, 10 μM NNK	2B1 catalyzed pyridine N-oxidation and α-methyl hydroxylation greater than α-methylene hydroxylation; no carbonyl reduction		*130*
3.	Rat	Lung, nasal mucosa	Antibody inhibition; chemical inhibition, 10 μM NNK	In lung: α-methylene hydroxylation inhibited by antibodies to 1A2 (46%), 2A1 (64%), 2B1 (23%); α-methyl hydroxylation inhibited by antibody to 2A1 (22%); NNK *N*-oxide and NNAL formation not affected. In nasal mucosa: α-methylene hydroxylation inhibited by antibodies to 1A2 (34%), 2A1 (20%); α-methyl hydroxylation inhibited by antibody to 1A2 (35%), 2A1 (7%)	In lung: No inhibition by antibodies to 1A1, 2C11, 2E1, 3A. In nasal mucosa: No inhibition by antibodies to 1A1, 2B1, 2C11, 2E1, 3A	*72*
4.	Rat	Lung, nasal mucosa	Antibody inhibition, 20 μM NNK	In lung: α-methylene hydroxylation inhibited by antibody to 2B1 and 2 (45%). In nasal mucosa: no inhibition	In lung: No inhibition of α-methylene hydroxylation by antibodies to 1A1, 2E1. In nasal mucosa: by 2B1 and 2, 1A1, 2E1	*71*
5.	Rat	Liver, lung, nasal mucosa	Chemical inhibition, 10 μM NNK	Effects of PEITC on hepatic NNK metabolism paralleled effects on PROD (for 2B1) and to a lesser extent EROD (for 1A) and erythromycin N-demethylase (3A); effects of PEITC on nasal mucosa NNK metabolism paralleled changes in EROD; effects of PEITC on pulmonary NNK metabolism did not parallel changes in EROD or PROD		*58*
6.	Rat	Liver, lung, nasal mucosa	Antibody inhibition, chemical inhibition, induction, 10 μM NNK	Hepatic α-methylene hydroxylation inhibited by antibodies to 1A2 (15%), 3A (13%); α-methyl hydroxylation inhibited by antibodies to 1A2 (19%), 2A1 (26%), 3A (13%); inducer studies generally consistent with these results; High K_ms for α-hydroxylation in liver, both control and induced, suggesting multiple constitutive and induced forms; no clear effects of inducer pretreatment on lung and nasal microsomal metabolism	2E1, 1A1, 2C11	*57*
7.	Rat		Ad293 cells transfected with rat CYP2B1 cDNA, 2.3 μM NNK	Pyridine N-oxidation and α-hydroxylation observed		*131*
8.	Rat	Liver, lung, nasal mucosa	Chemical inhibition, 10 μM NNK	PEITC and other arylalkyl isothiocyanates inhibited NNK oxidative metabolism: hepatic inhibition by PEITC similar to patterns with PROD, indicating 2B1 involvement; no correlation in lung		*59*
9.	Mouse	Lung	Antibody inhibition, 10 μM NNK	α-Methyl hydroxylation inhibited by antibodies to rat 1A1 (15%), 2B1 and 2 (25%); NNK *N*-oxide not inhibited	1A2	*108*
10.	Mouse	Lung	Antibody inhibition, 20 μM NNK	α-Methylene hydroxylation inhibited by antibodies to rat 2B1 and 2 (45%)	1A1, 2E1	*71*
11.	Mouse	Lung	Chemical inhibition, 10 μM NNK	PEITC had no effect on PROD and EROD but did inhibit α-hydroxylation and pyridine N-oxidation; benzyl isothiocyanate inhibited PROD and EROD, but had no effect on NNK metabolism		*59*
12.	Mouse	Lung	Antibody inhibition, 10 μM NNK	α-Methylene hydroxylation inhibited by antibody to rat 2B1 (23%), 1A2 (9%), 2A1 (47%), 2F (14%); α-methyl hydroxylation inhibited by 2A1 (22%); NNK *N*-oxide not inhibited	α-Methylene hydroxylation: 1A1, 2C11, 2E1, 3A. α-Methyl hydroxylation: 1A1, 2B1, 2C11, 2E1, 1A2, 3A, 2F. NNK-*N*-oxide: all of above and 2A1	*102*
13.	Monkey	Lung and liver	Antibody inhibition, chemical inhibition, 10 μM NNK	Inhibition of α-methylene hydroxylation and α-methyl hydroxylation by antibody to 2A6 in lung (12−16%) and liver (22−24%); inhibition of α-hydroxylation by α-naphthoflavone and coumarin		*115*
14.	Rabbit	Nasal mucosa	Reconstituted system, 2−155 μM NNK	NMa had more activity than NMb for α-methylene and α-methyl hydroxylation; no formation of NNK *N*-oxide or NNAL		*76*
15.	Human		Expressed P450s, mutagenicity, 0.05−0.7 mM NNK	Activation of NNK to a mutagen: 2A3 > 1A2 > 2D6 > 2E1		*132*
16.	Human	Liver	Microsomes and modeling		2D6	*133*

Table 3. (Continued)

Entry No.	Species	Tissue	Conditions	Main P450 Involvement	No Involvement	Reference
17.	Human	Liver, lung	Expressed P450s, microsomes, antibody inhibition, 10 μM−1 mM NNK	Methyl hydroxylation catalyzed by expressed 1A2 > 2A6, 2B7, 2E1, 2F1, 3A5; pyridine N-oxidation catalyzed by 2C8; no activity of 2C9, 2D6, 3A3, 3A4, 4B1; methyl hydroxylation in liver inhibited by antibodies against 1A2 and 2E1 and by 7,8-benzoflavone	No inhibition of α-methylene hydroxylation or pyridine N-oxidation in liver by antibodies to 1A2, 2E1, 2D1, 3A4, 2C8, 2A1; no inhibition of α-methyl hydroxylation in liver by antibodies to 2D1, 3A4, 2C8; no inhibition of any pathway in lung by these antibodies	*117*
18.	Human	Liver	Microsome mediated mutagenicity (2 mM NNK or NNAL), antibody inhibition, chemical inhibition, correlations	Mutagenicity of NNK and NNAL inhibited by antibodies to 2A6, 2E1, 1A2; NNK mutagenicity inhibited by diethyldithiocarbamate, 7,8-benzoflavone, and 4-methylpyrazole, but not quinidine; NNAL inhibited by diethyldithiocarbamate, 4-methylpyrazole, but not quinidine or 7,8-benzoflavone; NNK mutagenicity using reconstituted system with 1A2, 2A6, 2E1; NNAL, 1A2, 2A6, 2E1; NNK, but not NNAL mutagenicity correlated with coumarin 7-hydroxylation	No inhibition of mutagenicity of NNK or NNAL by antibodies to 3A4, $P450_{MP}$	*134*
19.	Human		Expressed P450, transformation, 10−190 μM NNK	α-Methylene hydroxylation and α-methyl hydroxylation catalyzed by microsomes from a human β-lymphoblastoid cell line expressing high levels of 2D6 cDNA; mutagenicity of NNK at the *hprt* locus		*135*
20.	Human		P450 2A6 expressed in C3H 10T1/2 cells, 1−3 mM NNK	Morphologic transformation by NNK indicating 2A6 catalyzes metabolic activation		*136, 137*
21.	Human		Lung microsomes, 10 μM NNK, chemical inhibitors, antibodies, P450-expressed microsomes	Inhibition of α-hydroxylation by α-naphthoflavone (1A) and coumarin (2A6); inhibition of α-hydroxylation by antibody to 2A6; high K_m for α-hydroxylation in 1A1-expressed microsomes; presence of a non-P450 pathway, possibly lipoxygenase	No inhibition by troleandomycin (3A) or quinidine (2D6)	*119*
22.	Human		Reconstituted system, 10 μM NNK	1A2 catalyzes α-methyl hydroxylation; inhibited by PEITC		*138*
23.	Human		Membranes from cells expressing P450s, liver microsomes, 1.2−500 μM NNK, correlations	α-Methylene hydroxylation catalyzed by 2A6 > 2E1, 2D6, 3A4; α-methyl hydroxylation catalyzed by 2D6 = 2A6 > 2E1 > 3A4; α-methylene hydroxylation correlated with 2A6 and 3A4 activity; α-methyl hydroxylation correlated with 1A2 activity; lowest K_m for 2A6, both α-hydroxylation pathways		*128*
24.	Human		Liver microsomes, correlations, 3 μM NNK or NNAL	α-Methylene hydroxylation and pyridine N-oxidation of NNK correlated with 3A4; α-methylene hydroxylation, α-methyl hydroxylation, pyridine N-oxidation, and reoxidation of NNAL correlated with 3A4		*118*
25.	Human		Expressed P450, 8 μM NNK	1A2 catalyzed α-hydroxylation; inhibited by sulindac and metabolites, but not by salicylates		*139*
26.	Human		Expressed P450	2B6 activates NNK to a mutagen efficiently		*140*

Table 4. Summary of P450 Involvement in NNK Metabolism[a]

	Rat			Mouse	Human	
	Liver	Lung	Nasal Mucosa	Lung	Liver	Lung
α-Methylene Hydroxylation	**1A2**, **2B1**, **3A**	**1A**, **2B1** 2A	**1A2**, **2A1**, **3A**	2A, 2B1 2F	**2A6**, **3A4** 2D6	
α-Methyl Hydroxylation	**1A2**, **2A1**, **3A** 2B1	2A	**1A2** 2A1	2A, 2B1	**1A2** 2A6, 3A4, 3A5, 2B6, 2B7, 2D6, 2E1, 2F1	**2A6**
Pyridine N-Oxidation	**2B1** 1A2, 3A				2C8, 3A4	

[a] Boldface, strong evidence; normal type, weak evidence. Criteria: reconstituted systems or expressed enzymes, antibody inhibition, chemical inhibition, induction, presence in tissue, correlation data, K_M values.

P450s 2A6 and 3A4 are implicated in NNK α-methylene hydroxylation in human liver. Since NNK concentrations will be low, and the K_m for P450 2A6 is lower than that of P450 3A4, P450 2A6 is probably more relevant to human exposure situations (*128*).

2.1.2.4. α-Methyl Hydroxylation. Rat hepatic P450 2B1 catalyzes conversion of NNK to keto alcohol in a reconstituted system. Keto alcohol formation is induced by pretreatment with PB; however, it is not inhibited by an antibody to P450 2B1 (*57*, *66*, *130*). Antibodies to P450s 1A2, 2A1, and 3A inhibit keto alcohol formation, and inducers of these enzymes increase its production (*57*).

An antibody to P4502A1 inhibits keto alcohol formation in rat lung, while in the nasal mucosa this reaction is inhibited most strongly by an antibody to P450 1A2 (*72*).

Table 5. In Vivo Metabolism of NNK[a]

Species	Reference
Rat	*46, 47, 64, 65, 145−156*
Hamster	*82, 95, 157*
Hamster, mouse, baboon	*158*
Mouse	*97, 98*
Monkey	*124, 159, 160*
Human	*124, 161−170*

[a] Condensed version; full table appears in Supporting Information.

α-Methyl hydroxylation is inhibited by antibodies to rat P450s 1A1, 2B1, and 2A1 in mouse lung (*102*, *108*). The effects of isothiocyanates on EROD and PROD activity do not correlate with their effects on α-methyl hydroxylation of NNK in rat or mouse lung (*58*, *59*).

P450 1A2 appears to be important in the production of keto alcohol in human liver (*117*, *138*). However, a variety of P450s catalyze this reaction including P450s 2A6 (with a high K_m), 2D6, 2B7, 2E1, 2F1, 3A4, and 3A5 (*128*).

2.1.2.5. Denitrosation. Enzyme involvement in NNK denitrosation has not been specifically investigated. It is probably a P450-mediated reaction (*54*, *129*, *143*, *144*).

2.1.2.6. ADP Adduct Formation. NNK(ADP)$^+$ and NNAL(ADP)$^+$ are apparently produced by microsomal NAD glycohydrolase. Rat liver and pancreatic microsomes catalyze these reactions, as does purified porcine brain NAD glycohydrolase (*62*).

2.1.3. In Vivo Metabolism of NNK and NNAL. Studies on in vivo metabolism of NNK are summarized in Table 5. Investigations have been carried out in rodents, primates, and humans. All studies in rodents and primates agree that NNK is rapidly distributed to most tissues and is rapidly metabolized (*82*, *97*, *154*, *159*, *160*). The three major routes of NNK metabolism—carbonyl reduction, pyridine N-oxidation, and α-hydroxylation—are consistently observed. There is no in vivo evidence for denitrosation or ADP adduct formation. The NNK−NNAL equilibrium favors NNAL in rodents, primates, and humans (*145*, *149*, *154*, *158*, *160*, *168*). α-Hydroxylation of NNK and NNAL occurs extensively in rat target tissues for carcinogenicity: nasal mucosa, lung, and liver (*154*). Accumulation of bound radioactivity is observed in these tissues due to protein and DNA binding (*47*, *82*, *97*). Relatively large amounts of unbound radioactivity are frequently associated with the stomach contents and melanin-containing tissues, due to the basicity of NNK and NNAL (*47*, *82*, *97*, *159*). Urine is the major route of excretion of NNK metabolites in all studies, with greater than 90% of the dose appearing in less than 24 h (*46*, *47*, *64*, *149*, *151*, *157*, *160*). Urinary metabolites vary with dose in rats and mice (*149*). For example, at higher doses, levels of NNAL and NNAL-Gluc are higher than at lower doses. Dose-related variations in levels of α-hydroxylation are also observed in studies with mice and rats (*149*).

2.1.3.1. Carbonyl Reduction. Pharmacokinetic studies demonstrate that NNAL is formed rapidly from NNK and that it predominates over NNK in blood (*145*, *158*, *160*). The half-life of NNAL in rats is 298 min, while that of NNK is 25 min (*145*). Similar results are obtained in other species (*158*, *160*). The discovery of the NNAL glucuronidation pathway was an important step forward (*149*). (*S*)-NNAL-Gluc predominates in rat urine, while in the patas monkey (*R*)-NNAL-Gluc is the major form in blood and urine (*124*). The (*R*)-NNAL-Gluc:(*S*)-NNAL-Gluc ratio is 1.9 in human urine, based on studies of Sudanese snuff (toombak) users (*124*, *164*). Mechanisms for selective formation of NNAL-Gluc diastereomers have not been worked out, as little is known about the pharmacokinetics and distribution of NNAL enantiomers. Urinary metabolites in mice and rats formed by the carbonyl reduction pathway include NNAL, NNAL *N*-oxide, and NNAL-Gluc. At low NNK doses, urinary levels of NNAL and NNAL-Gluc are low compared to metabolite levels from other pathways (*149*). NNAL-Gluc is the major metabolite in rat bile, but little if any reaches the feces (*150*). In contrast to the results in rodents, the patas monkey excretes substantial amounts of NNAL-Gluc in urine at low doses (*160*). This led to the development of methods to quantify NNAL-Gluc in human urine (*162*, *166*, *170*). Levels of NNAL-Gluc are generally greater than those of NNAL in human urine, and both exceed those of NNAL *N*-oxide (*168*). NNK and NNK *N*-oxide have not been detected in human urine (*166*, *168*). The ratio NNAL-Gluc:NNAL has been suggested as an index of detoxification of NNK in humans, and this ratio varies widely (*162*, *166*, *170*). Presently, we do not know if NNAL-Gluc is a detoxification product of NNAL, although metabolic studies indicate that only about 10% is ultimately converted to α-hydroxylation products in rats (*152*).

In smokers, excretion of NNAL plus NNAL-Gluc in urine averages 3.8 pmol/mg of creatinine (*166*). Based on excretion of 1.5 g of creatinine/24 h, total mean NNAL plus NNAL-Gluc is 5.6 nmol/24 h. From studies in the patas monkey, this represents about 20% of the NNK dose (*160*), indicating that the average uptake of NNK in smokers is 28 nmol/day, or 410 μmol (85 mg, 1.1 mg/kg) in 40 years. This estimate is similar to previous ones based on levels of NNK in cigarettes and is close to the lowest total dose of NNK (1.8 mg/kg) which induces lung tumors in rats, as discussed in section 2.6.1.1 (*32*, *33*).

The results of several studies demonstrate a significant correlation between total NNAL plus NNAL-Gluc and cotinine in human urine (*163*, *164*, *166*, *170*). Therefore, NNAL plus NNAL-Gluc appears to be an excellent indicator of lung carcinogen uptake, just as cotinine is an excellent indicator of nicotine uptake from tobacco exposure.

2.1.3.2. Pyridine Oxidation. Urinary NNK *N*-oxide and NNAL *N*-oxide generally comprise about 0−10% of the NNK dose in rodents (*46*, *64*, *149*, *151*, *160*). After low NNK doses in rodents and patas monkeys, levels of urinary pyridine *N*-oxides exceed those of NNAL and NNK (*168*). However, in humans, levels of urinary NNAL *N*-oxide are generally less than those of NNAL (*168*). Whereas pyridine N-oxidation appears to be an important detoxification route for NNK in rodents, it is relatively unimportant in humans (*168*). Glucuronidation of NNAL is likely the most important NNK detoxification pathway in humans (*162*, *166*, *168*).

6-HydroxyNNK is a minor urinary metabolite of NNK in rodents and monkeys, comprising about 1% of the dose (*151*). It is not detected in in vitro studies using microsomes, hepatocytes, or cultured tissues. Hydroxylation of the pyridine ring is observed in the bacterial metabolism of nicotine (*151*). Thus, 6-hydroxyNNK may be a product of bacterial transformation. Levels of 6-hydroxyNNK in rat urine are unaffected by pretreatment with PB, in contrast to NNK *N*-oxide and NNAL

N-oxide, which increase upon PB pretreatment probably due to induction of hepatic P450 2B1 (*64*). Therefore, 6-hydroxyNNK and NNK *N*-oxide are not formed from the same intermediate.

2.1.3.3. α-Hydroxylation. Consistently, keto acid and hydroxy acid are major urinary metabolites of NNK in rodents and primates (*46*, *64*, *149*, *151*, *157*, *160*). They are formed rapidly from NNK and are detected in the blood and various tissues (*154*). Keto acid is formed in the in vivo metabolism of keto alcohol (*171*), and it is also a likely oxidation product of keto aldehyde, although this does not seem to have been investigated. Therefore, the two α-hydroxylation pathways of NNK cannot be distinguished based on keto acid. Similarly, hydroxy acid is formed in NNAL metabolism by further oxidation of lactol and diol (*171*). Thus, the α-hydroxylation pathways of NNAL also cannot be distinguished by measuring hydroxy acid. Stereochemical studies indicate that hydroxy acid is formed by α-hydroxylation of NNAL, not by reduction of keto acid. Thus, metabolism of NNK in rats selectively produces (*S*)-hydroxy acid via (*S*)-NNAL, while metabolism of keto acid in rats produces mainly (*R*)-hydroxy acid (*172*).

Whereas the primary products of microsomal α-hydroxylation of NNK—keto alcohol and keto aldehyde—are not generally detected in the urine of NNK-treated animals, α-hydroxymethylNNK-Gluc (**1**) has been isolated from the urine of PB-pretreated rats (*64*). This important observation demonstrates unequivocally that α-hydroxymethylNNK (**2**), Figure 2, is the precursor to keto alcohol and that the product of α-hydroxylation has sufficient lifetime in vivo to be conjugated. Although the yield of this conjugate in urine is low (<1%), it is far higher than the percent of the dose that alkylates DNA. It has been suggested that the UDPGTs in the nuclear membrane could serve as a last line of defense against DNA pyridyloxobutylation by α-hydroxymethylNNK (*64*). α-HydroxymethylNNK-Gluc (**1**) could also be a transport form of activated NNK.

Urinary metabolites resulting from α-hydroxylation of NNK have not been quantified in humans. Keto acid and hydroxy acid are the major metabolites expected. Unfortunately, both are metabolites of nicotine, which occurs in concentrations about 3000 times as great as NNK in cigarette smoke. Studies in progress indicate that (*S*)-hydroxy acid is formed from NNK and NNN, while nicotine gives predominantly (*R*)-hydroxy acid via cotinine and keto acid. Therefore, (*S*)-hydroxy acid may be a potential indicator of NNK and NNN α-hydroxylation in humans (*172*). 3-Methyladenine is another urinary substance that could result from α-hydroxylation of NNK, via methylation of adenine in DNA followed by depurination or repair and excretion. There are mutliple sources of urinary 3-methyladenine including diet (*173*). Nevertheless, levels of 3-methyladenine in the urine of smokers are higher than in nonsmokers. Levels of 3-ethyladenine are also significantly elevated in smokers compared to nonsmokers, but the source of an ethylating agent in tobacco smoke is unclear (*173*−*176*).

2.2. DNA Binding of NNK and NNAL

2.2.1. Formation of Specific Adducts Derived from NNK

2.2.1.1. In Vitro Studies. Studies on the in vitro formation of specific NNK adducts are summarized in Table 6. All data, both in vitro and in vivo, as summarized below and in Figure 3, indicate that there are two major types of NNK−DNA adducts: methyl adducts formed by α-methylene hydroxylation and pyridyloxobutyl adducts formed by α-methyl hydroxylation. These results are consistent with the metabolism scheme outlined in Figure 2 and with expectations based on the well-established α-hydroxylation metabolic activation pathway of nitrosamines. The earlier studies of NNK DNA adducts focused on the methylation pathway since techniques were available to quantify the adducts produced and the miscoding properties of O^6-mG had been firmly established (*187*). Later investigations were able to obtain a more complete view as methods to quantify pyridyloxobutylation of DNA were developed and it became clear that the resulting adducts were mutagenic (*183*, *188*, *189*).

Table 6. NNK DNA Adducts In Vitro[a]

Species/System	Tissue	Reference
Rat	Nasal mucosa	*71*, *177*, *178*, *181*
	Lung, liver	*50*, *71*
	Oral, esophagus	*78*
Hamster	Lung	*91*
Mouse	Lung	*71*, *112*
S. typhimurium, G12, H3 cells		*183*
In vitro reactions		*179*, *180*, *182*, *184*−*186*

[a] Condensed version; full table appears in Supporting Information.

2.2.1.1.1. DNA Methylation (α-Methylene Hydroxylation). α-Methylene hydroxylation of NNK yields methane diazohydroxide (**7**) and/or the methyldiazonium ion (**11**), which reacts with DNA producing 7-mG, O^6-mG, and O^4-mT (Figure 3). Other adducts are probably also produced, based on studies of other methylating nitrosamines and nitrosoureas, but these have not been identified from NNK (*190*). The first studies quantified O^6-mdG in cultured rat nasal mucosa treated with NNK or NNAL; relatively high levels were detected by HPLC-immunoassay, consistent with the high activity in the nasal mucosa for α-hydroxylation of NNK (*177*, *178*). DNA methylation by NNK is observed in a number of in vitro studies with different systems capable of its metabolic activation, including rat lung cells and lung, liver, or nasal mucosal microsomes (with added DNA), rat oral tissue, and hamster lung (*50*, *71*, *78*, *91*).

2.2.1.1.2. DNA Pyridyloxobutylation (α-Methyl Hydroxylation). The chemistry of the intermediates resulting from α-methyl hydroxylation of NNK has been studied in some detail. Since α-hydroxymethylNNK (**2**; Figures 2−4) is not very stable, this metabolite has been generated in situ by solvolysis of NNKOAc (Figure 4). Similarly, CNPB (Figure 4) is a precursor to diazohydroxide **6** (*182*, *191*−*195*). Diazohydroxide **6** yields diazonium ion **10** which has three fates: reaction with nucleophiles (Y:) producing **14**, formation of the cyclic oxonium ion **13**, or loss of N_2 and $H^{\oplus}$ yielding the α,β-unsaturated ketone **15**. The latter two intermediates also react with nucleophiles to form products **16** and **17**. Extensive studies conclusively demonstrate that the major DNA adduct(s) formed by this pathway in vitro and in vivo, accounting for at least 50% of the DNA binding, releases keto alcohol (HPB; Figure 3) upon acid or neutral thermal, but not base, hydrolysis (*78*, *180*−*183*, *186*, *193*, *195*−*200*). This adduct(s) is produced via intermediates—**6**, **10**, and/or **13**—but not by HPB itself (*180*). The HPB-releasing adduct(s) have differing stablilties in DNA, being released in a triphasic manner

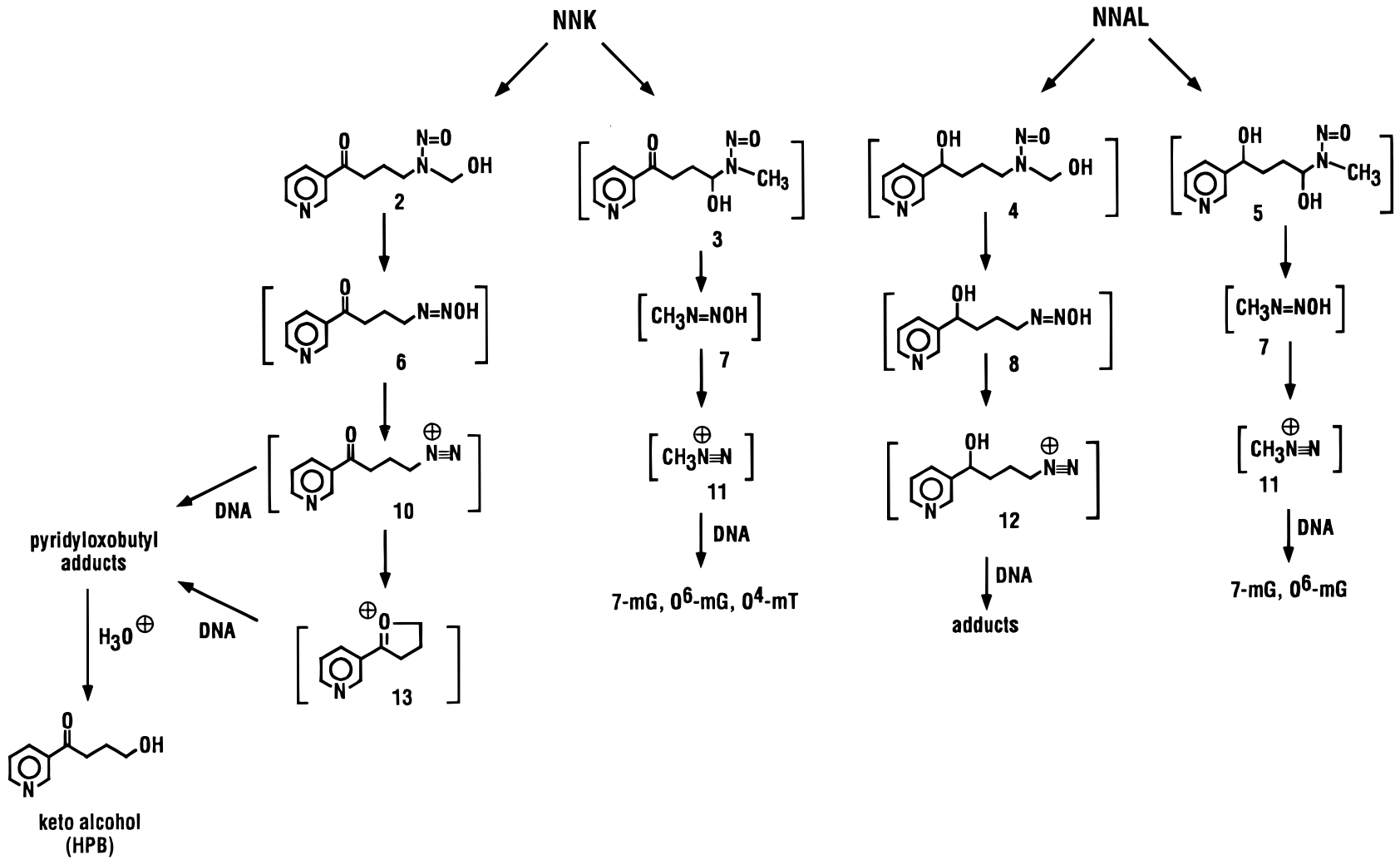

Figure 3. DNA adduct formation from NNK and NNAL as determined by in vivo studies in laboratory animals. NNAL adducts can also be formed via metabolic reconversion to NNK.

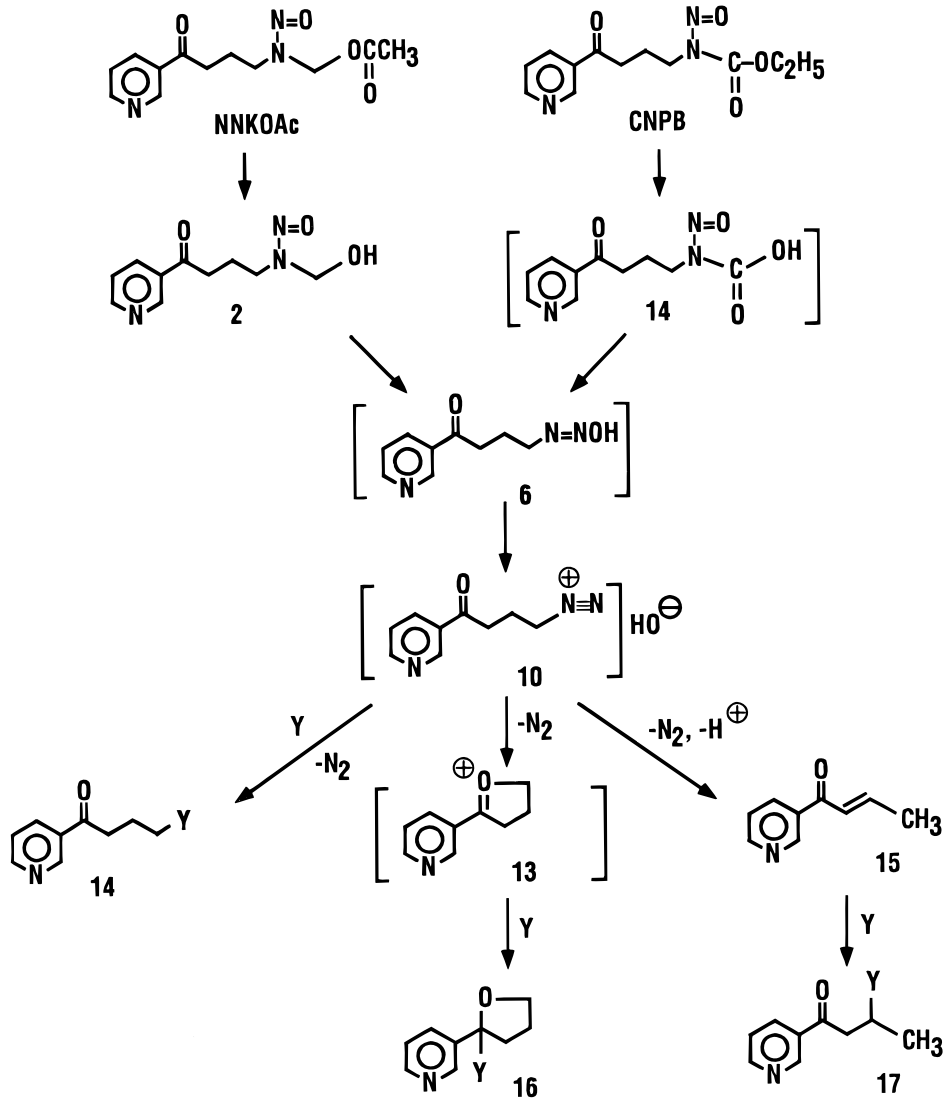

Figure 4. Intermediates and products formed upon solvolysis of NNKOAc and CNPB. When Y = OH, **16** ring-opens to **14**. Details are found in Spratt et al. (*194*).

(*182*). These adduct(s) do not appear to be stable at the nucleoside level or under the conventional HPLC conditions used for adduct analysis (*181*). The structure of the HPB-releasing adduct has not been determined. Unpublished data indicate that the major HPB-releasing adduct is a deoxyguanosine derivative.[2] The N^2 and O^6 adducts **18** and **20** of Figure 5 have been prepared (*181, 186*), but neither of these has properties consistent with the major HPB-releasing adduct; e.g., neither releases HPB upon neutral thermal hydrolysis, and both are stable under enzymatic hydrolysis and HPLC analysis conditions. Based on the chemistry described above, it appears likely that the HPB-releasing adduct(s) may be a pyridyltetrahydrofuryl-dG derivative **21** resulting from the reaction of N^2 with oxonium ion **13** and/or an acyclic 7-substituted pyridyloxobutyl-dG adduct **22** (Figure 5). Adducts from the reaction of oxonium ions with N^2 of dG have been characterized in reactions of α-acetoxy-*N*-nitrosopyrrolidine with dG or DNA, and similar chemistry may be occurring (*201, 202*). Moreover, the oxonium ion **13**, an S_N1-type reactant, is likely to bind at N^2 and/or O^6 of dG, while the diazonium ion **10**, an S_N2-type reactant, would be expected to bind preferentially at the 7-position of dG (*203*).

The identity of minor adducts formed by pyridyloxobutylation of DNA in vitro has been studied. The O^6-pyridyloxobutyl adduct **20**, Figure 5, is present in DNA reacted with NNKOAc (*186*). This is the only example of a structurally characterized DNA adduct resulting from methyl hydroxylation of NNK. Another minor adduct, formed upon incubation of NNK with cultured nasal mucosa, releases HPB upon acid hydrolysis but is not identical to the *N*^{2}-pyridyloxobutyl adduct **18**, Figure 5 (*181*). Reactions of CNPB, Figure 4, and the α,β-unsaturated ketone **15**, Figure 4, with dG yield the branched N^2 adduct **19**, Figure 5, but this has not been detected in tissues exposed to NNK in vitro or in vivo (*179, 181*).

Pyridyloxobutyl adducts inhibit AGT, the enzyme responsible for repair of O^6-mG. Since O^6-mG is also formed upon metabolic activation of NNK, this phenomenon is likely to be important with respect to the persistence of O^6-mG in NNK-exposed tissues (*204*). Studies with NNKOAc and oligonucleotides demonstrate that only pyridyloxobutylated poly(dGdC), not poly(dAdT), is able to prevent repair of O^6-mG by rat liver

[2]T. E. Spratt and S. S. Hecht, unpublished results.

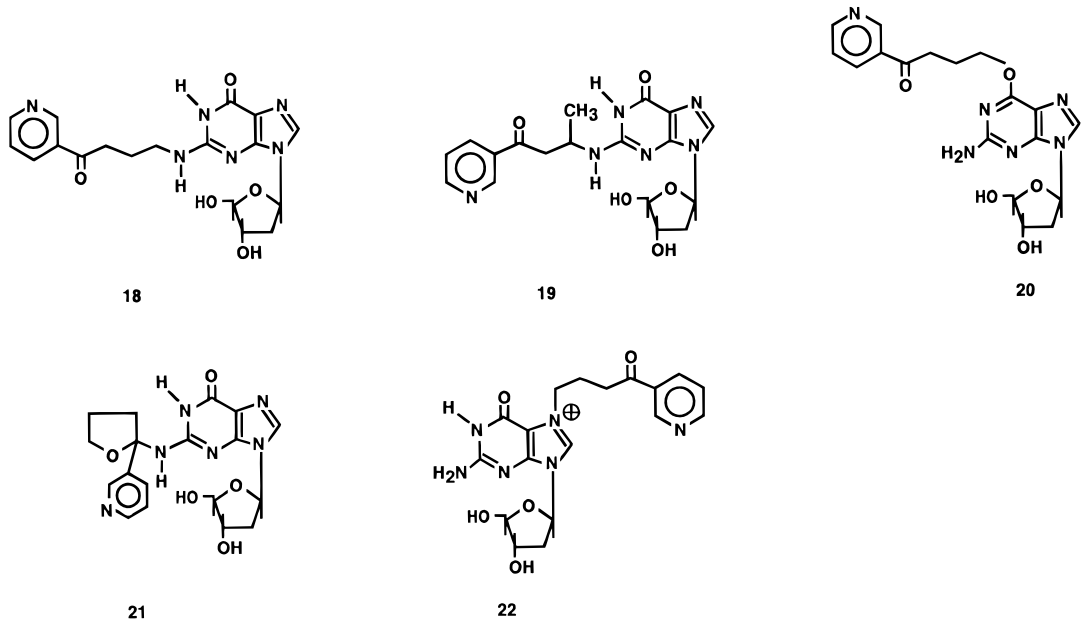

Figure 5. Known and potential DNA adducts of NNK formed by α-methyl hydroxylation: **18**, synthesized but not detected in hydrolysates of DNA from NNK-treated rats (*181*); **19**, formed by reaction of CNPB (Figure 4) or α,*β*-unsaturated ketone **15** (Figure 4) with dG but not detected in DNA from NNK-treated rats (*179, 181*); **20**, detected in DNA reacted with NNKOAc (*186*); **21**, **22**, potential structures of HPB-releasing DNA adducts, standards have not been prepared.

Table 7. NNK DNA Adducts In Vivo[a]

Species	Adduct type	Reference
Rat	7-mG, O^6-mG, O^4-mT	*49, 50, 55, 65, 146, 148, 177, 196, 199, 200, 205−218*
	Pyridyloxobutyl	*180−182, 196, 197, 199, 200, 211*
Hamster	7-mG, O^6-mG	*214, 216*
Mouse	7-mG, O^6-mG, O^4-mT	*98, 107, 112, 195, 213, 216, 218−225*
	Pyridyloxobutyl	*193, 195*

[a] Condensed version; full table appears in Supporting Information.

AGT. Furthermore, an oligomer containing dA, dT, and dG residues, but not its complement, reacts with NNK-OAc to generate an AGT-reactive adduct. Thus, the AGT-reactive adduct is an adduct with dG. Further studies show that the AGT-reactive dG adduct has a half-life of 1−2 weeks in DNA and represents a high percentage of the total HPB-releasing adducts in DNA (*185*).

2.2.1.2. In Vivo Studies. Investigations of NNK−DNA adduct formation in vivo are summarized in Tables 7 and 8. Since the first detection of O^6-mG and 7-mG in the liver and lung of NNK-treated F-344 rats in 1984 (*205*), there has been substantial research on the occurrence and biological significance of methyl and pyridyloxobutyl adducts resulting from metabolic activation of NNK. With few exceptions, adduct formation occurs principally in target tissues of NNK carcinogenicity: lung, nasal mucosa, and liver. Adduct measurements provide some important information on mechanisms of carcinogenesis by NNK, as well as giving some new insights on the relationship of NNK exposure to DNA adduct dose.

2.2.1.2.1. Rat Lung. Levels of 7-mG are 7.5−25 times as high as those of HPB released in whole lung, depending on dose, with lower ratios at lower doses (*197*). HPB-releasing adduct levels are about twice as great as levels of O^6-mG, and levels of O^6-mG are about 10 times as great as those of O^4-mT (*200, 206*). Consistently, the highest levels of O^6-mG and HPB-releasing adducts are found in the Clara cells of the rat lung, with lower amounts in type II cells, macrophages, and small cells (*50, 200, 207, 209, 212*). The dose response for adduct formation in whole lung and lung cell types is nonlinear (Figure 6). Adduct levels at the lowest doses are higher than would be expected by linear extrapolation from higher doses; e.g., alkylation efficiency increases dramatically at low doses (*50, 197, 200, 207, 212*). One interpretation of these data is the presence of P450s in the rat lung which efficiently catalyze α-hydroxylation of low concentrations of NNK (*50, 207, 212*). A second interpretation relates specifically to O^6-mG. Pyridyloxobutyl adduct concentrations increase at lower doses, perhaps leading to greater inhibition of AGT and consequent higher levels of O^6-mG (*204*).

During chronic treatment with high doses of NNK, O^6-mG increases consistently in the lung (*206*). At lower doses over a 4-day period, O^6-mG persists in Clara cells to a greater extent than in other cell types (*209*). This is partly because of lower AGT levels in the Clara cells than in other cell types after NNK treatment. NNK treatment inhibits AGT, which is probably due in part to DNA pyridyloxobutylation. However, when NNK is administered over the full 20-week course used for induction of lung tumors in rats, O^6-mG levels decrease by 82% in the Clara cells during the treatment period and are actually less than those in the macrophages at 20 weeks (*200*). The decrease is due to inhibition of P450-catalyzed α-methylene hydroxylation by NNK or one of its metabolites, possibly keto aldehyde, as demonstrated in a study of α-methylene hydroxylation by rat pulmonary microsomes isolated during 20 weeks of NNK treatment (*67*).

Structure−activity studies suggest that both DNA methylation and pyridyloxobutylation are important in NNK-induced lung tumorigenesis in the rat. Neither NDMA, which only methylates DNA, nor NNN, which pyridyloxobutylates but does not methylate DNA, is an effective lung carcinogen in the rat (*17, 146, 226*). NNK

Table 8. Summary of NNK−DNA Adduct Formation and Its Importance in Rodent Tumorigenesis

A. Comparison of Adduct Levels in Different Tissues

Tissue	Adduct levels[a,b]
F-344 rat lung	7-mG > **HPB-releasing adducts** > **O^6-mG** > O^4-mT
nasal mucosa	7-mG > O^6-mG > **HPB-releasing adducts**
liver	7-mG > HPB-releasing adducts > O^6-mG > O^4-mT
A/J mouse lung	7-mG > **O^6-mG** > HPB-releasing adducts > O^4-mT

B. Comparison of Tissue Levels of Different Adducts[c]

Adducts	Tissue levels in F-344 rats	Tissue levels in A/J mouse
7-mG	Nasal mucosa > lung[d] > liver[d]	Liver > lung
O^6-mG	Nasal mucosa > lung[d] > liver[d]	Liver > lung
O^4-mT	Liver > lung > nasal mucosa	NA[e]
HPB-releasing adducts	Lung[d] > liver[d] > nasal mucosa	Liver > lung

[a] Order may be different depending on cell type and time after dosing; see refs *195*, *197*, *200*, *206*, and *212*. [b] Boldface, important in NNK tumorigenicity. [c] See refs *146*, *197*, *200*, *206*, and *212*. [d] At low doses of NNK; order reversed at high doses (see Figure 6). [e] NA, not analyzed.

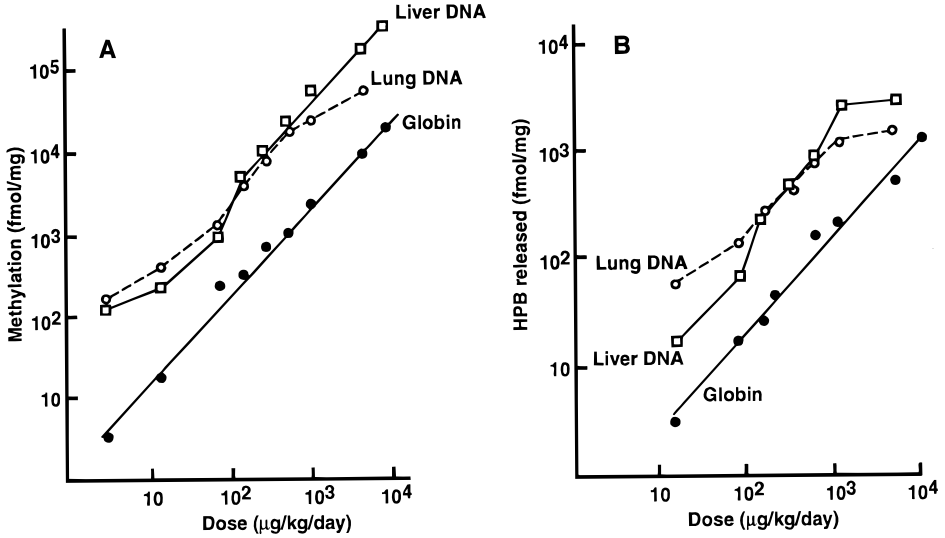

Figure 6. Plots of (A) levels of 7-mG in lung and liver DNA and total tritium in globin after treatment of rats with [*methyl*-D_3]NNK (3−10 000 μg/kg/day, ip) for 4 days or (B) HPB released from lung and liver DNA by acid hydrolysis or from globin by base hydrolysis. DNA and globin were obtained from rats treated with [5-^{3}H]NNK (15−10 000 μg/kg/day, ip) for 4 days. Details are found in Murphy et al. (*197*).

yields greater amounts of O^6-mG in Clara cells, but not type II cells, than does NDMA, and NNK is metabolized somewhat more effectively to a pyridyloxobutylating agent than is NNN in rat lung cell types (*50*, *69*). Such differences in binding and metabolism may partially account for the distinctly higher pulmonary carcinogenicity of NNK than of NDMA or NNN. However, only NNK can provide the mixture of DNA methylation and pyridyloxobutylation that appears to be critical for rat lung tumorigenesis. NNK-induced rat lung tumors arise in the type II cells (*212*). Levels of HPB-releasing adducts in the type II cells of NNK-treated rats correlate with lung tumor incidence over a range of doses, suggesting that pyridyloxobutylation is important (Figure 7A) (*200*). Levels of O^6-mG in Clara cells also correlate with lung tumor incidence over a wide dose range, suggesting some role of this adduct although the Clara cell is probably not the cell of origin of the tumors (Figure 7B) (*212*). Perhaps there is a signaling mechanism which becomes activated upon accumulation of O^6-mG in Clara cells but which has its ultimate effect on type II cells. The effects of PEITC on levels of HPB-releasing adducts in type II cells and other cell types of the lung correlate well with inhibition of NNK-induced lung tumorigenesis by PEITC, providing further evidence for the importance of HPB-releasing adducts (*200*). PEITC also inhibits O^6-mG levels in Clara cells to the same extent to which it inhibits lung tumorigenesis, but this is not seen in other cell types (*200*). Collectively, the available data indicate that HPB-releasing adducts and O^6-mG are both important in lung tumor induction by NNK in the rat.

2.2.1.2.2. Rat Nasal Mucosa. Levels of DNA methylation in the rat nasal mucosa are frequently higher than in other tissues of NNK-treated animals (*146*, *206*, *208*). This is a consequence of the high α-hydroxylation activity of the nasal mucosa. Although both α-methylene and α-methyl hydroxylations of NNK occur at similar rates in nasal mucosa microsomes (*72*), DNA methylation is greater than pyridyloxobutylation (*199*). For example, levels of methyl adducts are approximately 50−1000 times greater than those of HPB released, depending on dose and location in the nasal mucosa (*199*). These data indicate that, in the rat nasal mucosa, α-methylenehydroxyNNK (**3**) is more effective as a DNA-methylating agent than is α-hydroxymethylNNK (**2**) as a pyridyloxobutylating agent, possibly due to differences in reactivity of the resulting alkylating agents, or to other factors such as glucuronidation of α-hydroxymethylNNK. The differences between DNA methylation and pyridyloxobutylation are greater in the nasal mucosa than in the lung,

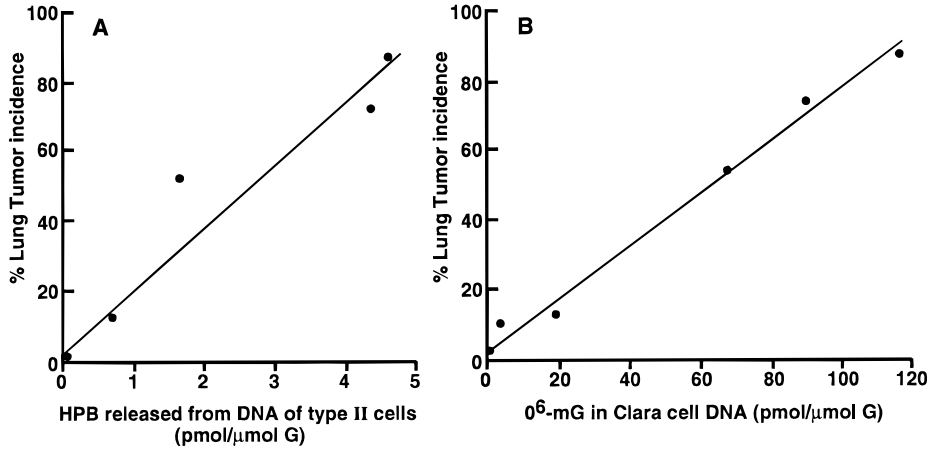

Figure 7. Correlation between DNA adduct levels and lung tumor incidence in NNK-treated F-344 rats: (A) HPB-releasing adducts in type II cells versus lung tumor incidence, $r = 0.97$ [from Staretz et al. (*200*)]; (B) O^6-mG in Clara cells versus lung tumor incidence, $r = 0.99$ [from Belinsky et al. (*212*)].

where levels of 7-mG are 7−25 times as great as those of HPB released and the latter exceed those of O^6-mG (*197, 200*). α-Methyl hydroxylation exceeds α-methylene hydroxylation in the lung, which partially explains the higher levels of HPB-releasing adducts in the lung than in the nasal mucosa (*72*). However, the data suggest that unknown factors—perhaps glucuronidation—in the rat nasal mucosa protect the DNA from α-hydroxymethyl-NNK.

Despite the relatively low levels of HPB-releasing adducts formed in the rat nasal mucosa upon treatment with NNK, these adducts appear to be important in tumor induction. NNK and NNN have similar carcinogenic activities toward the nasal mucosa, but NDMA has little activity (*146, 199*). NNK and NNN both pyridyloxobutylate nasal mucosa DNA, forming HPB-releasing adducts (*199*). NNN does not methylate DNA, and NDMA methylates, but does not pyridyloxobutylate, nasal DNA. These results support the role of DNA pyridyloxobutylation in rat nasal tumorigenesis. Studies with deuterated analogues of NNK further support this conclusion. Thus, [*methylene*-D_2]NNK is a stronger nasal carcinogen than is either [*methyl*-D_3]NNK or NNK (*199, 227*). Moreover, DNA pyridyloxobutylation by [*methylene*-D_2]NNK exceeds that of NNK, while levels of O^6-mG from [*methylene*-D_2]NNK are significantly less than those of NNK or [*methyl*-D_3]NNK (*199*). Collectively, these data provide strong support for the proposal that DNA pyridyloxobutylation is critical in rat nasal carcinogenesis by NNK. It has been proposed that O^6-mG in olfactory mucosa DNA, together with NNK-induced toxicity, is responsible for induction of nasal tumors (*208*). However, that study did not consider DNA pyridyloxobutylation.

2.2.1.2.3. Rat Liver. Levels of 7-mG are 13−49 times as great as those of HPB-releasing adducts in rat liver, and the latter are generally greater than those of O^6-mG (*197*). The 7-mG:HPB-releasing adduct ratio is lower at lower doses, as in the lung (*197*). At low doses, levels of HPB-releasing adducts are lower in liver than in lung (Figure 6B) (*197*). At high doses, the formation of 7-mG saturates in lung, but not in liver, whereas the formation of HPB-releasing adducts saturates in both tissues (Figure 6A,B) (*197*). The higher levels of HPB-releasing adducts than O^6-mG are probably due to differences in repair (*182, 206, 212*). During chronic NNK treatment, O^6-mG reaches a maximum in both hepatocytes and nonparenchymal cells, then declines rapidly due to induction of AGT (*206*). The removal of HPB-releasing adducts from hepatic DNA appears to be slower than that of O^6-mG (*182, 206*). The weak hepatocarcinogenicity of NNK is probably related to the efficient repair of O^6-mG.

2.2.1.2.4. Mouse Lung. Lung tumors are induced rapidly by a single dose of 10 μmol of NNK in the A/J mouse (*228*). This model has been used extensively to examine mechanistic questions as well as the modifying effects of chemopreventive agents. Levels of 7-mG are greater than those of O^6-mG which exceed those of HPB released (*195*). Levels of 7-mG and O^6-mG reach a maximum 4 h after injection of 10 μmol of NNK, whereas levels of HPB released are maximal after 24 h (*195*). Multiple P450s are involved in the α-hydroxylation of NNK in the mouse lung, and this probably contributes to the differing kinetics of methylation and pyridyloxobutylation (Tables 3 and 4). Levels of 7-mG and HPB released decrease with time, but O^6-mG is persistent, such that its levels exceed those of 7-mG 15 days after treatment (*195*). O^6-mG levels are highest in type II cells and Clara cells, followed by small cells and whole lung (*219*).

Persistent O^6-mG is the critical determinant of lung tumor induction in the A/J mouse (*195*) but does not account for differences in sensitivity to NNK-induced lung tumorigenesis between A/J and C57BL/6 mice (*225*). In A/J mice, AMMN, which can only methylate DNA, is highly tumorigenic whereas NNKOAc and NNN, which only pyridyloxobutylate DNA, are weakly active (*195*). Moreover, [*methylene*-D_2]NNK is significantly less tumorigenic than NNK or [*methyl*-D_3]NNK and also causes significantly less O^6-mG (*126*). There is an inflection in the dose−response curve for lung tumor induction by NNK in A/J mice, with an increase above a dose of 2−3 μmol of NNK, where persistent O^6-mG begins to be measurable (Figure 8) (*195*). Evidently, AGT activity is saturated above this dose. Levels of O^6-mG measured 96 h after treatment of A/J mice correlate strongly with tumor multiplicity, independent of the source of the methylating agent, e.g., NNK, AMMN, or AMMN plus NNKOAc (Figure 9) (*195*). In addition, GC-to-AT transitions in codon 12 of the *Kras* gene are observed in a high percentage of lung tumors induced by NNK in A/J mice, consistent with the importance of O^6-mG (*219*). Levels of O^6-mG in A/J mouse lung also correlate with the

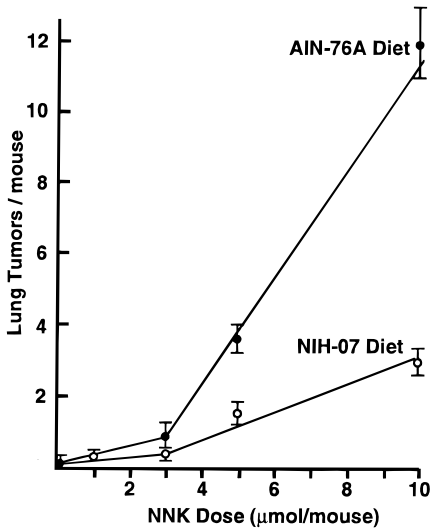

Figure 8. Tumor multiplicity ± SE in groups of 30 A/J mice given various single ip doses of NNK in saline and sacrificed 4 months later. Mice were maintained on either a AIN-76A diet (●) or NIH-07 diet (○). Details are found in Hecht et al. (*228*).

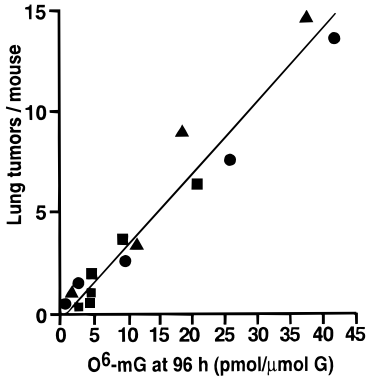

Figure 9. Relationship between lung tumor multiplicity and concentration of O^6-mG in lung DNA 96 h after treatment with NNK (■), AMMN (▲), or AMMN plus NNKOAc (●), $r = 0.98$. Details are found in Peterson and Hecht (*195*).

efficacy of isothiocyanates and indole-3-carbinol as inhibitors of tumorigenesis by NNK (*98, 107, 222*).

The pyridyloxobutylation pathway is important in increasing the activity of the methylation pathway in A/J mouse lung tumorigenesis since NNKOAc markedly increases the tumorigenicity of AMMN over a wide dose range (*195*). NNKOAc appears to enhance the persistence of O^6-mG in AMMN-treated mice due to the ability of HPB-releasing adducts to inhibit AGT (*185, 204*).

2.2.1.2.5. Mouse Liver. Adduct measurements have been made in A/J mouse liver after treatment with NNK, although tumor induction in this tissue is infrequent (*219*). As in the lung, the relative levels of adduct formation are 7-mG > O^6-mG > HPB-releasing adducts (*98, 193, 222*). Levels of HPB-releasing adducts are higher in liver than in lung, as are levels of 7-mG and O^6-mG (*98, 193, 195, 222*). The relatively high adduct levels in liver are consistent with metabolic studies showing efficient α-hydroxylation with little or no pyridine N-oxidation in this tissue, in contrast to lung where pyridine N-oxidation is a major competing detoxification pathway (Table 2). Despite the high DNA adduct levels in A/J mouse liver, tumors are observed in the lung and this is clearly related to susceptibility factors inherent in this mouse strain. There are no reports of NNK−DNA adduct levels in hepatic tissue of other mouse strains.

Table 9. Other Types of DNA Damage Induced by NNK and Metabolites[a]

System	Type of Damage	Reference
Rat hepatocytes	SSB	*51−53, 55, 235−238*
nasal mucosa	SSB	*237*
liver	SSB, 8-oxo-dG	*213, 240*
lung	8-oxo-dG	*213*
Hamster V79 cells	SSB	*235*
liver	SSB	*240, 242*
Mouse liver, lung	8-oxo-dG	*213, 223, 243*
Rabbit lung cells	SSB	*241*
Human lung	SSB	*239, 244*

[a] Condensed version; full table appears in Supporting Information.

2.2.1.2.6. Hamster Liver. Initial levels of 7-mG and O^6-mG are similar in rat and hamster liver after a single dose of NNK (*214*). However, O^6-mG is repaired more rapidly in the rat, $t_{1/2} = 12$ h, while in the hamster only 14% of the initial O^6-mG is repaired 72 h after treatment. 7-mG also persists longer in hamster than in rat liver. NNK rapidly depletes AGT in both rat and hamster liver, but it recovers in the rat and not in the hamster. These results do not correlate with tumor induction by NNK. Whereas NNK is a weak hepatocarcinogen in rats, it does not induce liver tumors in hamsters. The results suggest that O^6-mG is not important in hepatocarcinogenesis by NNK. Pyridyloxobutylation of hamster liver DNA by NNK has not been studied.

2.2.1.2.7. Adducts in Human Lung. Levels of 7-mG in human lung have been examined in several studies (*229−234*). In the largest study, the mean level of 7-mdG is $2.1/10^7$ nucleotides (*232*). This compares to a mean of $0.1/10^7$ nucleotides of HPB-releasing adducts (*198*), a 7-mdG:HPB ratio of 21, similar to the 7.5−25 ratio seen in rats (*197*). In another study, 7-mdG, but not HPB-releasing adducts, was detected (*233*). The origin of 7-mG in human lung is not clear, but two studies report higher levels in smokers than nonsmokers, suggesting that NNK is one source (*231, 234*). Another likely source is NDMA. A relationship is observed between debrisoquine extensive metabolizer genotype (*CYP2D6*) and higher 7-mdG adduct levels as well as between higher adduct levels and *CYP2E1* minor alleles, particularly at low cotinine levels (*232*). The significance of these findings is unclear at present, since P450s 2D6 and 2E1 do not appear to play a major role in NNK metabolism in the lung.

The detection of methyl and pyridyloxobutyl adducts in the lungs of smokers is consistent with the ability of human lung tissue to metabolically activate NNK by both α-hydroxylation pathways (*119*), but the quantitative aspects of the relationship of metabolism to levels of DNA adduct formation are unclear.

2.2.2. Other Types of DNA Damage. SSB are observed in hepatocytes incubated with NNK and in the livers of NNK-treated animals (Table 9). SSB are probably produced by spontaneous or enzymatic depurination of adducts such as 7-mG and 3-methyladenine, resulting from the DNA methylation pathway. There may be other sources as well. Among the metabolites of NNK, keto aldehyde has received the most attention as a source of SSB. However, the SSB induced by keto aldehyde appear to have different properties, for example, pH dependence, than those caused by NNK, indicating that the NNK SSB do not result from keto aldehyde (*238*). NNK SSB are

Table 10. Hemoglobin Binding of NNK[a]

Species	Reference
Rat	*155, 156, 193, 196, 197, 247−249*
Mouse	*193*
Human red cells in vitro	*60*
Human	*164, 250−253*

[a] Condensed version; full table appears in Supporting Information.

repaired slowly, with damage persisting for 2−3 weeks after a single NNK treatment in rats and hamsters (*242*).

NNK treatment causes increases in levels of the promutagenic adduct 8-oxo-dG in mouse and rat lung and in fetal liver following transplacental exposure of mice to NNK (*213, 243*). This may result from hydroxyl radicals or other reactive oxygen species generated during NNK metabolism. Decreases in levels of these adducts, but not O^6-mG, in the lungs of A/J mice occur upon treatment with inhibitors of NNK tumorigenesis, green tea, and its major antioxidant, EGCG (*223*). One study demonstrates an increase in NNK-induced SSB in human lung cells upon generation of superoxide (*239*). Collectively, these results suggest some role for oxidative damage in the pulmonary carcinogenicity of NNK.

2.3. Hemoglobin Binding of NNK and NNAL. Hemoglobin adducts of carcinogens are potentially useful as biomarkers of metabolic activation. Advantages of hemoglobin adducts over DNA adducts include the relative ease with which hemoglobin can be obtained in quantity, the lack of repair of adducts, and the relatively long lifetime, 120 days, of the red blood cell in humans, potentially allowing adduct accumulation (*245, 246*). With these potential advantages in mind, hemoglobin binding of NNK was investigated since it could provide a way for identifying those tobacco-exposed individuals who were particularly adept at activating NNK and thus could be at higher risk for cancer. Studies on hemoglobin binding of NNK are summarized in Table 10.

NNK forms globin adducts in rats by both the α-methylene and α-methyl hydroxylation metabolic activation pathways (*247*). α-Methylene hydroxylation leads to methylation of globin, while α-methyl hydroxylation pyridyloxobutylates globin. The methyl adducts have not been investigated extensively, because they would not be specific biomarkers of NNK metabolic activation. In contrast, only NNK and NNN among known tobacco product constituents produce the pyridyloxobutyl adducts. Depending on dose, species, and protocol employed, approximately 15−40% of the pyridyloxobutyl adducts are released as HPB upon mild base hydrolysis (*60, 193, 197, 247*). Less material is released upon acid treatment (*247, 249*). The HPB-releasing adducts do not form upon treatment of hemoglobin or rodents with HPB but are formed from NNK and stable precursors to α-hydroxymethylNNK (*193*). The HPB-releasing adducts have been identified by studies with $[^{18}O]H_2O$ as carboxylic acid esters, most likely with glutamate or aspartate (*249*). The reactivity of pyridyloxobutylating agents with *N*-acetylcysteine has been investigated to determine the potential role of cysteine in the formation of the pyridyloxobutyl adducts that do not release HPB, e.g., 60−85% of the bound material (*248*). CNPB (Figure 4) reacts with *N*-acetylcysteine to give cyclic, branched, and straight chain adducts resulting from the addition of sulfur to the three major derived electrophiles—diazonium ion **10**, oxonium ion **13**, and α,β-unsaturated ketone **15**—of Figure 4 (*248*). Hydrolysis of globin from NNK-treated rats shows that pyridyloxobutyl cysteine adducts are not formed in detectable amounts (*248*). The structures of the non-HPB-releasing pyridyloxobutyl adducts in globin have not been determined.

Coincubation of rat hepatocytes and human red blood cells results in the formation of HPB-releasing adducts, as well as pyridyloxobutyl adducts that do not release HPB, as seen in vivo (*60*). The HPB-releasing adducts are not formed in the absence of hepatocytes. Therefore, α-hydroxymethylNNK (**2**), or perhaps its glucuronide **1** (or possibly the corresponding diazohydroxide **6** or diazonium ion **10**), is stable enough to migrate out of the hepatocyte and into the red blood cell, where pyridyloxobutylation of globin occurs. α-HydroxymethylNNK-Gluc (**1**) is the most likely candidate for the transported metabolite leading to HPB-releasing adducts in globin, but this has not been investigated. Interestingly, red blood cells themselves are able to activate NNK producing globin adducts, but not the HPB-releasing adducts (*60*). Part of this activation process occurs by heme-catalyzed α-methylene hydroxylation of NNK resulting in formation of keto aldehyde, which probably forms lysine adducts in globin, and methane diazohydroxide (**7**), which methylates histidine and cysteine.

If hemoglobin adducts are to be useful surrogates for DNA adducts, there must be a predictable relationship between their formation. In rats, both methyl and pyridyloxobutyl hemoglobin adducts increase linearly over greater than a 3000-fold dose range (Figure 6A,B) (*197*). DNA adducts, both methyl and pyridyloxobutyl, also increase over this range, but the relationship to dose is nonlinear. As discussed above, DNA adducts at low doses are higher than would be expected by extrapolation from higher doses. At the highest doses examined, DNA adduct levels saturate. Thus, the relationship between hemoglobin adducts and DNA adducts of NNK in rats is somewhat complex. Comparable studies in humans have not been performed. The utility of the HPB releasing adduct as a biomarker of overall carcinogenic effect of NNK has been demonstrated in two studies in rats. Treatment of rats with PEITC or PHITC and NNK results in a significant decrease of HPB-releasing adducts over an 18-month period, compared to rats treated with NNK only, and, concurrently, a significant decrease in lung tumor induction in the rats treated with isothiocyanates and NNK (Figure 10) (*155, 156*).

The mild base-catalyzed release of HPB from hemoglobin is advantageous for the development of analytical methods because it provides a way of separating the analyte, HPB, from the remaining globin. The released HPB can be purified, derivatized as its pentafluorobenzoate, and detected with high sensitivity by GC/negative ion chemical ionization-MS (*250, 252, 253*). These studies show that levels of HPB-releasing adducts in the globin of smokers are considerably lower than levels of adducts formed from other tobacco smoke and environmental carcinogens such as BaP and 4-aminobiphenyl (*250, 252, 254*). For example, HPB-releasing hemoglobin adducts are typically 60−80 fmol/g in smokers, whereas hemoglobin adducts of BaP are 2600 fmol/g, and those of 4-aminobiphenyl are about 1000 fmol/g. The comparatively low levels of HPB-releasing hemoglobin adducts are probably a consequence of the relative instability of α-hydroxymethylNNK (**2**) compared to the reactive metabolites formed from BaP and 4-aminobiphenyl. The low

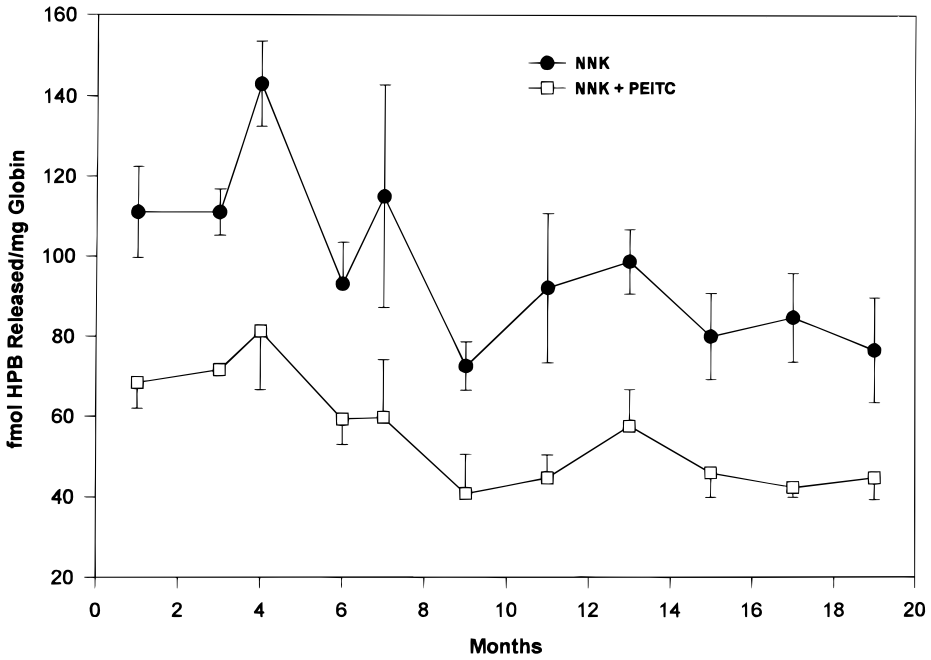

Figure 10. Levels of HPB-releasing hemoglobin adducts in rats treated chronically with 2 ppm NNK in the drinking water and NIH-07 diet (●) or with 2 ppm NNK in the drinking water and NIH-07 diet containing 489 ppm PEITC (□). Values are means for three rats per group at each time point; bars, SD [from Hecht et al. (*156*)].

Table 11. Comparison of Biomarkers of Tobacco-Specific Nitrosamine Uptake and Metabolic Activation

Species	NNAL Plus NNAL-Gluc in Urine	NNAL *N*-Oxide in Urine	HPB-releasing DNA Adducts	HPB-releasing Hb Adducts
Rat	More prevalent at higher than at lower doses of NNK; modified in a predictable way by chemopreventive agents (Table 5; section 2.1.3.1)	Present to a greater extent than NNAL or NNAL-Gluc at lower doses (Table 5; section 2.1.3.1)	Readily quantifiable; important in lung and nasal tumorigenesis (Tables 7, 8; section 2.2.1.2)	Readily quantifiable; correlate with DNA adducts; good biomarker of risk; mechanism of formation unclear (Table 10; section 2.3)
Human	Readily quantifiable; good marker of NNK uptake; correlates with urinary cotinine; modified in predictable way by chemopreventive agents; NNAL-Gluc/NNAL ratio may indicate NNK detoxification (Table 5; section 2.1.3.1)	Minor metabolite, < than NNAL (Table 5; section 2.1.3.1)	Quantifiable by GC/MS, but limited data available (section 2.2.1.2.7)	Quantifiable by GC/MS, levels frequently near background (Table 10; section 2.3)

levels of HPB-releasing adducts found in smokers limit the utility of this assay as a biomarker, because adduct levels are frequently not much higher than assay background amounts. HPB-releasing adducts are higher in snuff-dippers and nasal snuff users than in smokers (*164*, *250*, *252*). This may be due to differences in pharmacokinetics between inhaled NNK/NNN vs orally or nasally administered substance. Or, other constituents of tobacco smoke, not present in snuff, may inhibit α-methyl hydroxylation of NNK or 2′-hydroxylation of NNN.

Biomarkers of tobacco-specific nitrosamine uptake and metabolic activation currently employed in human studies are summarized in Table 11, with comparison to data obtained in rats. At present, NNAL and NNAL-Gluc in urine are the most useful biomarkers employed in human studies.

2.4. Mutations in the *Kras* and *p53* Genes from NNK-Induced Lung Tumors

2.4.1. Mouse. Mutations in the *Kras* gene are commonly found in mouse lung tumors induced by NNK. These studies are summarized in Table 12. By far the most common mutation is a GGT → GAT transition in codon 12. This is seen in multiple strains of mice and occurs in a high percentage of the lung tumors as well as in hyperplasias (*219*, *221*, *224*, *225*, *257*). This G → A transition mutation is consistent with the important role of O^6-mG in NNK-induced lung tumorigenesis in the mouse, as summarized above. Predominantly GGT → GAT mutations are also present in tumors induced by pure methylating agents such as AMMN (*189*). The pyridyloxobutylation pathway of NNK metabolic activation also produces mutations in codon 12 of *Kras*, but the spectrum is different from that of NNK and AMMN. Thus, the pure pyridyloxobutylating agent NNKOAc causes GGT → TGT and GGT → GTT mutations in codon 12 in addition to the GGT → GAT mutations (*189*).

While the induction of G → A mutations in codon 12 of *Kras* appears to be consistent with the O^6-mG pathway of NNK metabolic activation, there are evidently other factors involved. For example, only 2/22 lung tumors induced by NNK in the relatively insensitive C57BL/6 mouse have *Kras* mutations (*225*). In another study, treatment of mice with NNK followed by BHT increases lung tumor induction compared to NNK alone but decreases the frequency of GGT → GAT mutations in codon 12 of *Kras* (*256*). The frequency of activation of *Kras* and the GGT → GAT mutation in codon 12 are not affected by the time after NNK treatment, nor is there a correlation between the proliferative activity of the lung lesions and the presence of the mutations. Thus, *Kras* gene mutations appear to play a minor role in the selective growth advantage of NNK-induced lung lesions

Table 12. Specific Mutations in Tumors and Other Systems after NNK Treatment

Entry No.	Sex/Strain/Species or System	NNK Dose	Conclusions	Reference
1.	Female A/J mouse	0.24 mmol/kg i.p. 3 x weekly, 7 weeks	7/11 lung adenocarcinoma with GGT→GAT mutations in codon 12 of *Kras*; similar results with NDMA	*219*
2.	Male C3H/HeJ mouse	0.24 mmol/kg i.p. 3 x weekly, 7 weeks	11/11 lung tumors with GGT→GAT mutations in codon 12 of *Kras*; similar results with NDMA; difference in susceptibility between A/J and C3H/HeJ mouse not due to differences in *Kras* activation	*221*
3.	Female A/J mouse	0.5 mmol/kg i.p., once	In hyperplasias, 85% of the *Kras* mutations were GGT→GAT in codon 12, suggesting that the mutations are due to O^6-mG	*224*
4.	Female C57BL/6 mouse	0.5 mmol/kg i.p. once or 72 μmol/kg weekly, 24 weeks	Only 2/22 activated *Kras* in lung tumors; no *p53* mutations; alternate pathways involved in tumor induction	*225*
5.	Female A/J mouse	0.25 mmol/kg i.p., 3 x weekly, 7.3 weeks; NNKOAc, 0.13 mmol/kg, 3 x weekly, 5.6 weeks; AMMN, 0.038 mmol/kg, 3 x weekly, 1.3 weeks	26/28 NNK lung tumors with a GGT→GAT mutation in codon 12 of *Kras*; 8/21 NNKOAc tumors with GGT→GAT, 5/21 with GGT→TGT, 4/21 with GGT→GTT; 18/18 AMMN tumors with GGT→GAT	*189*
6.	Female A/J mouse	0.53 mmol/kg i.p., once	46/59 lung tumors with a GGT→GAT mutation in codon 12 of *Kras*	*255*
7.	Female A/J mouse	1.54 mmol/kg total p.o. over 7 weeks	19/19 lung tumors with a GGT→GAT mutation in codon 12 of *Kras*; 11/34 such mutations in mice treated with NNK followed by dietary butylated hydroxytoluene, which increased tumor multiplicity	*256*
8.	Female A/J mouse and (A/JxTSG-*p53*)F_1 mouse	0.5 mmol/kg i.p., once	15/17 lung tumors with a GGT→GAT mutation in codon 12 of *Kras* in A/J mice; 20/23 in (A/JxTSG-*p53*)F_1 mice; tumor multiplicity inhibited by PEITC, but induced tumors had same incidence of activated *Kras* and same mutations; no *p53* mutations observed	*257*
9.	Female A/J mouse	0.48 mmol/kg i.p., once	45/72 lung tumors with a GGT→GAT mutation in codon 12 of *Kras*; no difference with varying histological types	*258*
10.	Male Syrian golden hamster	0.12 mmol/kg s.c. 3 x weekly, 20 weeks	3/6 lung tumors with GGT→GAT mutation in codon 12 of *Kras*; no mutations in *p53*	*259*
11.	Male Syrian golden hamster	0.048 mmol/kg s.c., 3 x weekly, 25 weeks	49/75 lung tumors with a GGT→GAT mutation in codon 12 of *Kras*; 15/75 with GGC→GAC in codon 13; 1/24 with mutations in *p53*	*260*
12.	*E. coli* strain NR3835	5 mM NNK and 9,000 x g supernatant from Aroclor pretreated male Sprague−Dawley rats	GC→AT transitions account for 55% of the mutations; multiple other base substitutions implying mutation induction due to methylation and pyridyloxobutylation	*261*
13.	Male Swiss-Webster mouse with *E. coli* NR3835	0.12 mmol/kg once	GC→AT transitions account for 94% of the mutations, consistent with O^6-mG as their source	*262*
14.	Chinese hamster ovary AS52 cell line lipofected with human P450 2A6	5.8 mM NNK	21 clones with deletions/rearrangements, 77 with point mutations; 81% GC→AT transitions; 4 of 6 GC→AT hot spots at the second G of GGT	*263*

in the A/J mouse (*258*).

No mutations have been detected in the *p53* gene from NNK-induced mouse lung tumors (*225, 255, 257*).[3]

2.4.2. Hamster. As in the mouse, a high prevalence of GGT → GAT mutations in codon 12 of *Kras* is observed in lung tumors induced by NNK in Syrian golden hamsters (*259, 260*). No data are available concerning the importance of O^6-mG in hamster lung tumorigenesis induced by NNK, but the *Kras* results do suggest that O^6-mG may be important.

Only 1 of 24 tumors examined had a mutation in the *p53* gene (*260*).

2.4.3. Rat. There is no evidence for *Kras* or *p53* mutations in lung tumors induced by NNK in rats (*264*).[3]

[3]Z. Ronai and S. S. Hecht, unpublished results.

2.5. Possible Relationship of Mutations in NNK-Induced Lung Tumors in Rodents to Mutations in Human Lung Tumors. Mutations in codon 12 of the *KRAS* gene are present in 24−50% of human primary adenocarcinomas but are rarely seen in other types of lung tumors (*265*−*267*). These mutations are more common in smokers and ex-smokers than in nonsmokers, suggesting that they may be induced by a component of tobacco smoke (*268*). The most frequently observed mutation is GGT → TGT, typically representing about 60% of the mutations in codon 12, followed by GGT → GAT (20%) and GGT → GTT (15%). The prevalence of G → T mutations has led to speculation that they may be due to BaP, which can induce such mutations through the diol epoxide metabolic activation pathway (*268, 269*). However, G → T mutations are also induced by NNKOAc

Table 13. NNK Tumorigenicity[a]

Strain/Species	Route	Target Organs	Reference
F-344 rat	s.c.	Lung, nasal cavity, liver	*17, 146, 208, 210−212, 226, 227, 280, 281*
	oral swab (with NNN)	Lung, oral cavity	*274*
	oral swab	Lung	*277*
	p.o.	Lung, liver, nasal cavity, pancreas	*127, 155, 156, 278*
	intravesicular	Lung, liver	*275*
	gavage	Liver, lung, nasal cavity	*276, 279*
A/J mouse	i.p.	Lung	*16, 98, 100, 101, 106, 107, 112, 113, 189, 195, 219, 220, 222, 224, 225, 228, 255, 257, 258, 283, 285−288, 290, 294, 295, 297−299, 301−309*
	p.o.	Lung	*109, 114, 139, 256, 292, 293, 296*
	gavage	Lung, forestomach	*223, 289, 303*
	i.p. (gestation)	Lung (progeny)	*287*
Sencar mouse	topical	Skin, lung	*282*
Swiss mouse	oral	Lung, forestomach	*284*
	i.p.	Lung, liver	*287, 291, 300*
	i.p. (gestation)	Liver (progeny)	*287, 300*
BALB/c mouse	oral	Lung, liver, stomach	*284, 311*
$C3B6F_1$ mouse	i.p.	Lung, liver	*287*
	i.p. (gestation)	Liver (progeny)	*287*
C3H/HeJ mouse	i.p.	Lung	*221*
C57BL/6 mouse	i.p.	Lung	*225*
$(A/J \times TSG\text{-}p53)F_1$ mouse	i.p.	Lung	*257*
Syrian golden hamster	s.c.	Lung, nasal cavity, trachea	*157, 259, 260, 310, 313, 319*
	topical, cheek pouch	Lung, forestomach, nasal cavity, trachea	unpublished, *311, 314, 318*
	s.c. (gestation)	Respiratory tract, adrenal (progeny)	*312, 317*
	i.t. (gestation)	Nasal cavity, adrenal, pancreas (progeny)	*315, 316*
	p.o.	None (promotion of pancreatic tumors)	*316, 320*
Mink	s.c.	nasal cavity, forebrain, lung, liver	*321*

[a] Condensed version; full table appears in Supporting Information.

(*189*). In the mouse, the O^6-mG pathway of NNK metabolic activation is clearly the major one involved in tumor induction, and this is consistent with the high percentage of GGT → GAT mutations in the *Kras* gene isolated from mouse lung tumors induced by NNK. But in the rat, both the pyridyloxobutylation and methylation pathways are critical in lung tumorigenesis by NNK. We do not know the relative importance of these pathways in human lung. If pyridyloxobutylation is critical, as in the rat, a higher percentage of G → T transversions would be expected as a result of NNK exposure than is observed in mice. The attempt to assign mutations in human genes to particular carcinogen adducts, in the absence of other information, may be futile when considering an exposure as complex as tobacco smoke. Numerous DNA-damaging compounds are present in tobacco smoke, and many of these cause G → T transversion mutations: examples, in addition to nitrosamines and PAH, include aromatic amines, oxygen radicals, and α,*β*-unsaturated aldehydes (*270−273*).

2.6. Carcinogenicity of NNK and NNAL

2.6.1. Rat

2.6.1.1. Lung. Lung is the main target of NNK carcinogenicity in the rat (Tables 13 and 14). Thus, lung tumors are induced in F-344 rats when NNK is given in the drinking water, by sc injection, gavage, oral swabbing, or intravesicular administration. Lung tumors are always induced preferentially over local tumors; e.g., few if any oral cavity or esophageal tumors are observed upon oral swabbing or administration in the drinking water, and no subcutaneous or bladder tumors are seen upon sc or intravesicular administration (*17, 127, 275, 277*). Male rats appear to be more susceptible than females (*17, 226*). Clearly, the lung tumors result from systemic absorption of NNK. In dose−response studies, lung tumors are prevalent at lower doses, where they are often observed to the virtual exclusion of other tumor types (*127, 212*). The lung tumors are predominantly adenomas and adenocarcinomas, with lower incidences of adenosquamous and squamous cell carcinomas. Evidence for progression from hyperplasia to adenoma to adenocarcinoma has been presented. Ultrastructural studies indicate that the tumors arise in the type II cells of the rat lung (*212*).

Table 14. Induction of Lung Tumors by NNK

Species and Strain	Route
Mouse	
A/J	i.p., gavage, p.o.
Sencar	skin
BALB/c	oral
Swiss	oral, i.p.
$C3B6F_1$	i.p.
C3H/HeJ	i.p.
C57BL/6	i.p.
$(A/J \times TSG\text{-}p53)F_1$	i.p.
F-344 rat	s.c., p.o., oral swab, gavage, intravesicular
Syrian golden hamster	s.c., application to cheek pouch
Mink	s.c.

The lowest total dose of NNK shown to induce lung tumors in rats is 1.8 mg/kg (8.7 μmol/kg) which induced a 6.7% lung tumor incidence and a 16.4% incidence of hyperplasia in a group of 60 rats; these results were significant as a trend in this dose−response study (*212*). In the same study, a total dose of 6 mg/kg induced a 10% tumor incidence and a 15% incidence of hyperplasia. In a later study however, the 6 mg/kg dose induced significant pulmonary hyperplasia only (*280*).

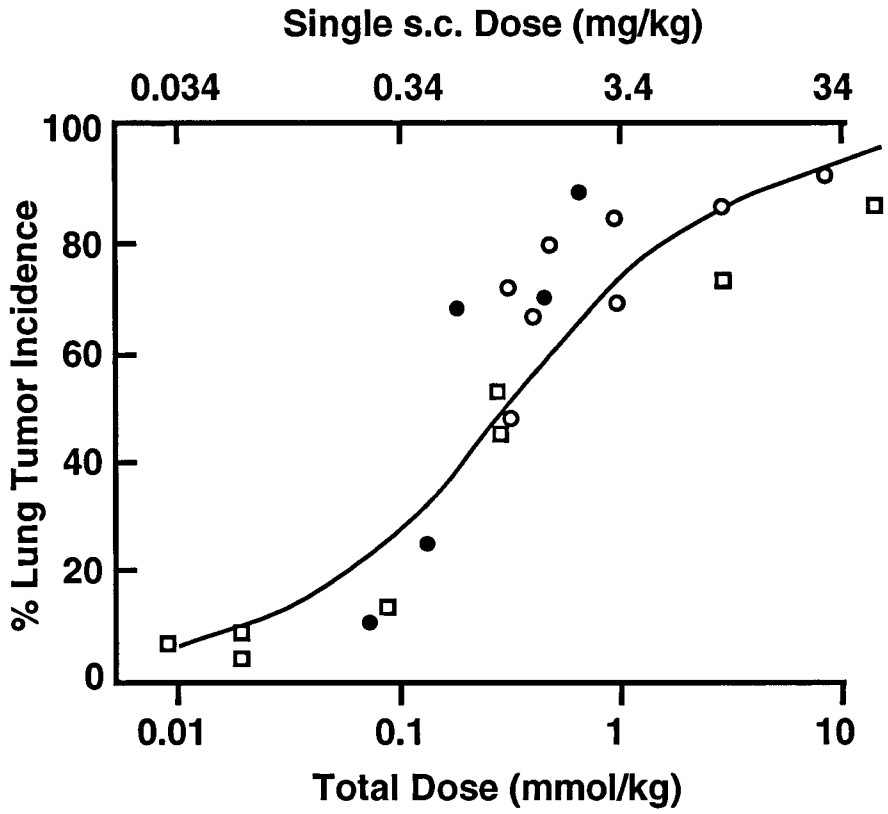

Figure 11. Relationship between dose of NNK and lung tumor incidence in male F-344 rats. Data combined from protocols using sc injection, 3 times weekly for 20 weeks, in studies carried out by investigators at NIEHS (□) (*212, 280*) or the American Health Foundation (○) (*146, 211, 226, 227, 281*) or by administration in the drinking water (●) (*127, 156, 278*).

Dose−response data for lung tumor induction from 10 studies of NNK carcinogenicity in rats are summarized in Figure 11. Seven of these studies (designated by the symbol ○ or □) were carried out by the standard sc injection protocol in which rats are treated 3 times weekly for 20 weeks, then sacrificed after 2 years. In the other three studies, NNK was administered for life in the drinking water (studies are indicated by the symbol ●). Several points are noteworthy. The TD_{50} corresponds to a total dose of 0.3 mmol/kg, which is equivalent to a single dose of 1 mg/kg in the sc injection protocol. A sharp increase in tumor induction occurs between total doses of approximately 0.1−0.3 mmol/kg, corresponding to single doses of 0.3−1 mg/kg sc. A similar increase in O^6-mG formation in Clara cells occurs between single sc doses of 0.3−1 mg/kg, suggesting some critical role for O^6-mG in Clara cells in lung tumor induction, probably in combination with pyridyloxobutylation of type II cells, as discussed in section 2.2.1.2.1 (*200, 212*). The seven sc studies summarized in Figure 11 were carried out in two different institutions. Six of the seven employed the NIH-07 diet, and one used the AIN-76A diet. The three drinking water studies gave similar tumor yields as the sc studies, despite the major difference in protocols. Collectively, these data demonstrate that the NNK rat lung tumor induction model is robust and reproducible.

NNAL and NNK administered in the drinking water (5.0 ppm for life) give almost identical incidences of lung tumors: 87% and 90%. Thus NNAL is clearly not a detoxification product of NNK in the rat (*127*). Comparing NNK and NNN, a different picture emerges: lung tumors are seen almost exclusively in the NNK-treated rats (*17, 226*). Comparison of NNK and NDMA shows that NNK is a more potent pulmonary carcinogen than NDMA (*146*). There are no differences in lung tumor incidence among NNK, [*methylene*-D_2]NNK, and [*methyl*-D_3]NNK (*227*). Collectively, these data are consistent with the hypothesis that both DNA methylation and pyridyloxobutylation are necessary for lung tumor induction by NNK in the rat.

As indicated above, lung tumor incidence is the same in rats maintained on a cereal-based NIH-07 diet vs a semisynthetic AIN-76A diet (*281*). This contrasts to observations in A/J mice, discussed below. On the other hand, a high-fat diet (23.5% fat in the AIN-76A diet) enhances lung tumorigenesis by NNK in rats compared to rats maintained on 5% fat (*278*), but this is not observed in A/J mice.[4]

Transient increases in 8-oxo-dG occur in the lungs of NNK-treated rats, suggesting that oxidative DNA damage may play a role in pulmonary carcinogenesis by NNK (*213*). However, *N*-acetylcysteine, an antioxidant, does not inhibit lung tumor induction by NNK, nor is lung tumor induction enhanced by coadministration of ozone (*280, 281*). These results do not support a role for oxidative damage in lung tumor induction by NNK in the rat.

2.6.1.2. Nasal Cavity. Nasal tumors are the second most common neoplasm in rats treated by sc injection with NNK. They are observed in a high percentage of the animals treated with total doses of 3, 1, or 0.3 mmol/kg, but not at lower doses (*146, 212, 226, 227*). In contrast, NNK given in the drinking water induces few nasal tumors at a total dose of 0.68 mmol/kg indicating that hepatic clearance of orally administered NNK may

[4]S. S. Hecht, unpublished results.

decrease its effect in the nose (*127*). The malignant nasal tumors, seen at the highest doses, are mainly olfactory neuroblastomas. At lower doses, benign tumors of the respiratory region occur. It has been suggested that *Aspergillus* infection of the nasal passages may contribute to mortality in NNK-treated rats (*281*).

As with the lung tumors, structure−activity studies are informative. NNK and NNN have similar carcinogenic activities toward the rat nose, but NDMA has little activity (*146, 199*). In comparative studies, [*methylene*-D_2]NNK is a more potent nasal carcinogen than is NNK or [*methyl*-D_3]NNK, and it also pyridyloxobutylates olfactory DNA more effectively than NNK or [*methyl*-D_3]NNK (*199, 227*). In contrast, O^6-mG formation is significantly lower in nasal mucosal DNA of rats treated with [*methylene*-D_2]NNK than with NNK (*199*). Collectively, these results strongly support the hypothesis that pyridyloxobutylation of DNA is the critical lesion for induction of nasal tumors by NNK in the rat.

2.6.1.3. Liver. Liver tumors are routinely observed in rats treated by sc injection with NNK at total doses of 3 mmol/kg or higher, but not at the lower doses which produce lung and nasal tumors (*212, 226*). At high doses, hepatocellular carcinomas and hemangiosarcomas are observed (*17, 212*). Malignant liver tumors have not been produced in bioassays of NNK administered in the drinking water. Comparative studies by sc injection indicate similar potency for NNK and NDMA, but little activity for NNN. These results suggest that DNA methylation may be important in liver tumorigenesis by NNK, although AGT induction results in low levels of hepatic O^6-mG even at higher doses (*212, 214*). Toxicity and other factors may contribute to liver tumor induction by NNK (*212*).

2.6.1.4. Pancreas. NNK induces a low level of exocrine pancreatic tumors when administered in the drinking water. Incidences are generally of borderline statistical significance (*127, 156, 278*). The tumors are predominantly acinar adenoma and adenocarcinoma. Some ductal tumors have also been observed. Pancreatic tumors are larger in animals maintained on a high-fat diet (*278*). NNAL is significantly more tumorigenic than NNK to the rat exocrine pancreas (*127*). NNAL causes acinar adenomas and adenocarcinomas, as well as ductal adenocarcinomas. NNK and NNAL are the only tobacco constituents known to induce exocrine pancreatic tumors and may be involved as causative agents for cancer of the pancreas in smokers (*33*). About 25% of pancreatic cancer is caused by smoking (*30*).

2.6.2. Mouse. Sensitive and resistant mouse strains develop lung tumors when treated with NNK, although tumor incidence and multiplicity in the resistant strains are lower and the tumors require a longer time to develop (Tables 13 and 14). Liver and forestomach tumors are seen occasionally, depending on the protocol. The A/J mouse, a sensitive strain, has been used extensively for studies of lung tumor induction by NNK and the effects of modifiers, notably chemopreventive agents. The most commonly used model is a single ip dose of 10 μmol of NNK (*228*). This results in 7−12 lung tumors/mouse after 16 weeks. These adenomas eventually progress to adenocarcinomas which are the predominant lesions 50 weeks after injection (*224*). The assay is rapid, reproducible, and lends itself to quantitation. Another advantage is the precision of the dose. The single-dose assay is useful not only for screening of chemopreventive agents but also for mechanistic studies since the initiation and promotion/progression stages of carcinogenesis are clearly separated. A second assay that has been fairly extensively used involves po administration of NNK for 7 weeks, such that the total dose is 44 μmol (*296, 309*). An advantage of this assay is that chronic administration more closely mimics the human situation. However, the NNK-treated mice lose weight, and the stages of carcinogenesis cannot be separated as readily as in the single-dose model. Forestomach tumors are also observed in A/J mice treated by multiple gavage (*289*).

Hyperplasias are present along the alveolar septa 14 weeks after a single ip dose of 10 μmol of NNK (*224*). These appear to arise from the type II cells. Progressively with time, the frequency of hyperplasias decreases with an increase in the frequency of adenomas, about 50% of which arise within the hyperplasias. Carcinomas increase in frequency 34 weeks after NNK treatment, eventually comprising 50% of the pulmonary lesions. About 30% of these arise within adenomas (*224*). The well-characterized single-dose model has also been used to study chemotherapeutic efficacy (*297*).

Dose−response studies in A/J mice demonstrate that there is an inflection in the dose−tumor multiplicity curve at about 2−3 μmol, with a sharp increase in lung tumor multiplicity at higher doses (Figure 8) (*195, 228*). Persistent O^6-mG is detectable after a single dose of 2.5 μmol of NNK, but not at lower doses, suggesting that the inflection in the dose−response curve is due to saturation of AGT (*195*). The critical role of O^6-mG in lung tumor induction by NNK in the A/J mouse is strongly indicated by the correlation between levels of persistent O^6-mG and lung tumor multiplicity (Figure 9) (*195*). The central role of O^6-mG is also supported by the lower tumorigenicity of [*methylene*-D_2]NNK than NNK or [*methyl*-D_3]NNK; [*methylene*-D_2]NNK also produces significantly lower amounts of O^6-mG in lung DNA than the other two compounds. Moreover, G → A mutations in codon 12 of the *Kras* gene are found in a high percentage of NNK-induced lung tumors, which is also consistent with the role of O^6-mG (Table 11). In contrast to the rat, pyridyloxobutylation of DNA does not appear to play a direct role in lung tumor induction in A/J mice but rather acts as a cocarcinogen by inhibiting AGT, thus enhancing levels of O^6-mG (*195, 204*).

Mice maintained on a semisynthetic AIN-76A diet have significantly higher tumor multiplicities after a single dose of NNK than do mice maintained on the cereal-based NIH-07 diet (Figure 8) (*228*). This contrasts to results in rats. Further studies demonstrate that this effect is a result of dietary modification of tumor initiation by NNK.[3] Apparently, there are inhibitors of NNK−DNA binding present in the NIH-07 diet; these could be vegetable constituents.

Depending on the protocol employed, NNAL has approximately 30−70% as much activity as NNK for lung tumor induction in A/J mice (*106, 126, 301*), while in rats, NNAL and NNK are equipotent lung tumorigens (*127*). Comparative studies demonstrate that NNK is the most potent of the tobacco-specific nitrosamines as a lung tumorigen in A/J mice (*16, 106, 195, 283, 286, 301*). NNK is unusual among nitrosamines because it can induce tumors of the skin in Sencar mice when applied topically; lung tumors are also observed (*282*).

NNK is a weak transplacental tumorigen in A/J, $C3B6F_1$, and Swiss mice inducing lung or liver tumors

in the progeny (*287*). In Swiss mice, Aroclor is a promoter of transplacental lung and liver tumorigenesis in males (*300*). In neonatal Swiss mice, NNK is a weak lung and liver tumorigen, and these are also promoted by Aroclor (*291, 300*).

2.6.3. Hamster. Lung, trachea, and nasal cavity are the main target tissues of NNK administered sc or applied to the cheek pouch (Table 13). These respiratory tract tumors are induced by a number of different protocols including a single dose of 1 mg of NNK (*157, 260, 310, 313, 319*). The lung tumors are adenomas and adenocarcinomas predominantly, with some squamous and adenosquamous carcinomas (*157, 260, 319*). The tracheal tumors are multiple papillomas, sometimes obliterating the tracheal lumen (*157*). The nasal cavity tumors can be aggressive and are of a mixed olfactory, respiratory type (*157*). One report describes the induction of neuroendocrine tumors of the lung in animals treated with NNK and hyperoxia, but this has not been confirmed (*260, 313, 319*).

Administration of NNK in the drinking water, at doses of 1 or 3 ppm, does not lead to tumors of any type (*316*). Similar results are obtained with NNAL, 2 or 5 ppm (*320*). This contrasts to the sensitivity of the rat to tumor induction by orally administered NNK, at doses of 0.5−5 ppm, or NNAL, at 5 ppm (*127, 156*). One difference is that female hamsters were used in these experiments versus male rats. The apparent lower response of hamsters to NNK carcinogenicity in these experiments could also relate to higher hepatic clearance in the hamster than in the rat, since hamster liver microsomes are more effective in NNK metabolism than are rat liver microsomes (*63*). Liver tumors are not observed in hamsters treated by any NNK protocol. A protective role of AGT in this resistance to NNK has been excluded (*214*).

NNAL enhances pancreatic adenocarcinoma induced by BOP in hamsters, and NNK may also have a slight enhancing effect (*316*). Since NNAL and NNK appear to have little if any genotoxic activity in the rat pancreas, their abilities to induce pancreatic tumors in rats could relate to a promoting or indirect DNA-damaging effect.

NNK is a potent transplacental carcinogen in the hamster, inducing tumors at a variety of sites in the offspring. After sc injection of NNK during gestation, tumors of the respiratory tract and adrenal glands are observed (*312, 317*), while intratracheal administration results in tumors of the nasal cavity, adrenal glands, and pancreas. A high incidence of ductular adenocarcinomas of the pancreas occurs in the female offspring of hamsters treated with NNK and ethanol (*315*).

2.7. Inhibition of NNK-Induced Lung Tumorigenesis. Chemoprevention of lung cancer by inhibiting the carcinogenicity of tobacco smoke compounds is a potential way to reduce lung cancer incidence in smokers who are addicted to nicotine and cannot stop even after taking part in smoking cessation programs (*322*). Therefore, many agents have been tested for their ability to inhibit lung tumorigenesis by NNK. Inhibitors of NNK lung carcinogenicity are summarized in Table 15.

Inhibitors have been tested most extensively in the A/J mouse model (Table 15A−D). Among compounds administered by gavage before NNK administration, isothiocyanates are the most potent inhibitors known. Doses of certain isothiocyanates as low as 0.04−0.1 μmol will inhibit lung tumor induction by a single dose of 10 μmol of NNK (Table 14) (*302*). Isothiocyanate inhibition of lung tumor induction by NNK depends on lipophilicity and reactivity with glutathione. Higher isothiocyanate lipophilicity is correlated with inhibitory activity, while higher reactivity with glutathione decreases inhibitory potency (*302*). Thus, 10-phenyldecylITC is a much more potent inhibitor of NNK-induced lung tumorigenesis in the mouse than is PEITC (*302*). The isothiocyanate functional group is necessary for inhibition (*113*). Extensive studies demonstrate that inhibition of P450s by isothiocyanates is the major mechanism by which they inhibit NNK-induced lung tumorigenesis in the mouse (Table 1) (*59, 102, 108*). This results in inhibition of O^6-mG formation and tumorigenesis (*107, 222*). When added to microsomal incubations, PEITC inhibits NNK oxidation by competitive and noncompetitive mechanisms (*102, 108*). Longer chain arylalkyl isothiocyanates are stronger inhibitors of NNK metabolic activation than is PEITC, which correlates with the tumor inhibition data (*59, 102*). For example, 6-phenylhexylITC is a potent competitive inhibitor of NNK oxidation in mouse lung microsomes with an apparent K_i of 11−16 nM (*59*). Dietary PEITC has significant effects on P450 enzymes in the mouse, but little effect on phase II enzymes (*59, 102*). In the mouse, dietary PEITC and other isothiocyanates have differing effects on P450 activities depending on the protocol employed, the dose, and the time after dosing (*59, 102, 108*). In general, strong inhibitory effects are observed on pulmonary NNK metabolic activation, but the inhibition does not correlate with the effects of the isothiocyanates on specific P450 enzymes such as P450 2B1, 2E1, or 1A (*59, 102, 108*). These results suggest that there are unknown P450 enzymes present in the mouse lung which metabolically activate NNK and are inhibited by isothiocyanates.

Other inhibitors of NNK-induced lung tumorigenesis in the mouse include indole-3-carbinol, green and black tea and their components, diallyl sulfide, hydroxyalkanones related to ipomeanol, *p*-XSC, arylalkynes, ellagic acid, BHA, D-limonene, and nonstereoidal antiinflammatory drugs such as sulindac, ibuprofen, and piroxicam. All of these compounds, as well as the isothiocyanates, are effective when given before, before and during, or before, during, and after NNK administration to mice. Only green and black tea extracts, lovastatin, and *p*-XSC are effective as inhibitors when given after NNK administration, although it is likely that *myo*-inositol and dexamethasone have similar activity (*305*).

Indole-3-carbinol inhibits NNK-induced mouse lung tumorigenesis by increasing hepatic clearance of NNK, probably by induction of a P450 1A. Thus, less NNK reaches the lung (*98*). A similar mechanism may occur in humans, as treatment of smokers with indole-3-carbinol decreases levels of NNAL plus NNAL-Gluc in urine, consistent with increased hepatic clearance of NNK (*169*). Green and black tea as well as EGCG inhibit NNK oxidation and NNK-induced DNA methylation in vitro, but not methylation of pulmonary DNA in vivo (*112, 223*). However, green tea and EGCG suppress the formation of 8-oxo-dG in the lungs of mice treated with NNK (*223*). Black tea and its polyphenols have antiproliferative activity which is important in the inhibition of NNK tumorigenesis by tea given after NNK treatment (*308*). Among the other inhibitors of NNK tumorigenicity active when given before or during NNK treatment, inhibition of NNK metabolic activation is likely to be the

Table 15. Inhibitors of NNK-Induced Lung Tumorigenesis in Mice and Rats

A. Compounds or Mixtures That Inhibit NNK-Induced Lung Tumorigenesis in A/J Mice When Given Before, Before and During, or Before, During, and After NNK Treatment

Entry No.	Compound or Mixture	Protocol type[a]	Lowest total gavage dose (μmol) or concentration in diet or drinking water shown to significantly inhibit lung tumor multiplicity	Reference
1.	PEITC	A	5	*295, 304*
		C	80 ppm in diet	
2.	3-PhenylpropylITC	A	0.2	*222*
3.	4-PhenylbutylITC	A	0.2	*222*
4.	5-PhenylpentylITC	A	0.2	*222*
5.	6-PhenylhexylITC	A	0.1	*222*
6.	8-PhenyloctylITC	A	0.1	*302*
7.	10-PhenyldecylITC	A	0.04	*302*
8.	1-HexylITC	A	1	*302*
9.	2-HexylITC	A	1	*302*
10.	1-DodecylITC	A	1	*302*
11.	2,2-DiphenylITC	A	1	*302*
12.	1,2-DiphenylITC	A	1	*302*
13.	Indole-3-carbinol	A	100	*98, 304*
		C	1800 ppm in diet	
14.	Decaffeinated green tea extract	B	6000 ppm in drinking water	*294*
15.	Decaffeinated black tea extract	B	6000 ppm in drinking water	*294*
16.	Green tea	F	20,000 ppm in drinking water	*223*
17.	EGCG	F	560 ppm in drinking water	*223*
18.	Caffeine	F	1120 ppm in drinking water	*223*
19.	Decaffeinated green tea	A, B	6000 ppm in drinking water	*112*
20.	Diallyl sulfide	A	105	*101*
21.	4-Hydroxy-1-phenyl-1-pentanone	A	25	*306*
22.	7-Hydroxy-1-phenyl-1-octanone	A	25	*306*
23.	Amylbenzene	A	25	*306*
24.	4-Hydroxy-1-phenylpentane	A	25	*306*
25.	*p*-XSC	C	5 ppm Se in diet	*298, 323*
		B	7.5 ppm Se in diet	
26.	5-Phenyl-1-pentyne	A	5	*299*
27.	4-Phenyl-1-butyne	A	5	*299*
28.	2-Ethynylnaphthalene	A	5	*299*
29.	Ellagic acid	D	250 ppm in diet	*296*
30.	BHA	D	5000 ppm in diet	*114*
31.	Sulindac	D	60 ppm in diet	*309*
32.	Sulindac	E	123 ppm in diet	*293*
33.	Ibuprofen	D	263 ppm in diet	*293*
34.	Piroxicam	D	25 ppm in diet	*293*
35.	Aspirin	D	294 ppm in diet	*139*
36.	D-Limonene	A, C, G	184	*289, 304*
			6300 ppm in diet	
37.	Lemon or orange oil	A, G	25 mg	*289*
38.	*myo*-Inositol	H	10,000 ppm in diet	*305*
39.	Dexamethasone	H	0.5 ppm in diet	*305*

B. Compounds or Mixtures That Fail To Inhibit NNK-Induced Lung Tumorigenesis in A/J Mice When Given Before, Before and During, or Before, During, and After NNK Treatment

Entry No.	Compound or Mixture	Protocol type	total gavage dose (μmol) or concentration in diet or drinking water	Reference
1.	PhenylITC	A	20	*107*
2.	BITC	A	20	*107*
3.	4-Oxo-4-(3-pyridyl)butylITC	A	20	*107*
4.	4-(3-Pyridyl)butylITC	A	4	*222*
5.	AllylITC	A	5	*302*
6.	4-Ipomeanol	A	1	*100*
7.	4-Hydroxy-1-(2-thienyl)-1-pentanone	A	25	*100*
8.	4-Hydroxy-1-(3-pyridyl)-1-pentanone	A	25	*100*
9.	1,4-Diphenyl-4-hydroxy-1-butanone	A	25	*306*
10.	Sodium selenite	D	5 ppm Se in diet	*298*
11.	1-Dodecanol	A	1	*113*
12.	Dodecane	A	1	*113*
13.	Naproxen	E	230 ppm in diet	*293*
14.	Oltipraz	E	250 ppm in diet	*109*
15.	Sulindac	A, D	123 ppm in diet	*309*
16.	Esculetin	E	2300 ppm in diet	*296*
17.	Esculin	E	4600 ppm in diet	*296*
18.	β-Carotene + retinol	E	2100 + 900 ppm in diet	*292*
19.	Selenomethionine	B	3.75 ppm Se in diet	*323*

Table 15. (Continued)

C. Compounds or Mixtures That Inhibit NNK-Induced Lung Tumorigenesis in A/J Mice When Given After NNK Treatment

Entry No.	Compound or Mixture	Protocol type	Lowest concentration in diet or drinking water shown to significantly inhibit lung tumor multiplicity	Reference
1.	Decaffeinated green tea extract	I	3000 ppm in drinking water	*294*
2.	Decaffeinated black tea extract	I	3000 ppm in drinking water	*294*
3.	Decaffeinated green tea	I	6000 ppm in drinking water	*112*
4.	Lovastatin	I	400 ppm in diet	*324*
5.	Black tea solids	J	3000−6000 ppm in drinking water	*308*
6.	*p*-XSC	I	7.5 ppm Se in diet	*323*

D. Compounds or Mixtures That Fail To Inhibit NNK-Induced Lung Tumorigenesis in A/J Mice When Given After NNK Treatment

Entry No.	Compound or Mixture	Protocol type	Concentration in diet (ppm)	Reference
1.	PEITC	I	489	*288*
2.	BITC	I	149	*288*
3.	Sulindac	K	123	*293*
4.	Selenomethionine	I	3.75 as Se	*323*

E. Compounds That Inhibit NNK-Induced Lung Tumorigenesis in F-344 Rats

Entry No.	Compound	Protocol type	Concentration in diet (ppm)	Reference
1.	PEITC	L	489	*211*
2.	PEITC	M	652, 1304	*281*
3.	6-PhenylhexylITC	M	438, 876	*281*
4.	PEITC	N	489	*156*
5.	6-PhenylhexylITC	N	219	*155*

F. Compounds That Fail To Inhibit NNK-Induced Lung Tumorigenesis in F-344 Rats

Entry No.	Compound	Protocol type	Concentration in diet (ppm)	Reference
1.	Sinigrin	L	1245	*210*
2.	N-Acetylcysteine	M	6528, 13,056	*281*

[a] Protocol types: A, compound given before a single dose of 10 μmol of NNK; B, compound given before, during, and 1 week after a single dose of 10 μmol of NNK; C, compound given before, during, and after NNK until sacrifice (NNK, a single dose of 10 μmol); D, compound given before, during, and after NNK, until sacrifice (NNK, 0.3 μmol in drinking water for 7 weeks to total dose of 44 μmol); E, compound given during NNK administration, 0.3 μmol in drinking water for 7 weeks to total dose of 44 μmol; F, compound given before and during NNK (56 μmol/kg) administered by gavage, 3 times weekly for 10 weeks; G, compound given before 2.4 μmol of NNK twice weekly for 8 weeks; H, compound given before, during, and after NNK, until sacrifice (NNK, 7.7 μmol weekly for 2 weeks); I, compound given after NNK until sacrifice (NNK, a single dose of 10 μmol); J, compound given from week 16−52 after a single dose of NNK, 10 μmol; K, compound given after NNK administration until sacrifice (NNK, 0.3 μmol in drinking water for 7 weeks to total dose of 44 μmol); L, compound given in diet 1 week before and during 20 weeks of NNK sc injections, 3 times weekly, 8.5 μmol/kg; M, compound given in diet 1 week before, during, and 1 week after 20 weeks of NNK sc injections, 3 times weekly, 7.2 μmol/kg; N, compound given in diet 1 week before and during 111 weeks of NNK administered in the drinking water, 2 ppm.

predominant mechanism of action, although the effects may not be as strong as observed with isothiocyanates (*100, 101, 104, 105*). An exception is the nonsteroidal antiinflammatory drugs, notably sulindac. Sulindac has little or no effect on NNK metabolism or on 8-oxo-dG formation in NNK-treated mice (*109, 325*). However, it attenuates NNK-induced immunosuppression. Chemoprevention by these drugs may be mediated through modulation of prostaglandin E_2 synthesis (*309, 326*).

A smaller number of compounds have been tested in rats because such bioassays are more costly. The isothiocyanates PEITC and 6-phenylhexylITC are effective inhibitors of NNK-induced lung tumorigenesis, while sinigrin and *N*-acetylcysteine are inactive (Table 15E,F). Inhibition of tumorigenesis by PEITC in the rat lung is selective; no effects are observed on tumors of the liver and nasal cavity induced by NNK (*211, 281*).

The predominant mechanism by which isothiocyanates inhibit NNK tumorigenesis in rat lung is selective inhibition of P450-mediated NNK metabolic activation in the lung. As in mice, isothiocyanate inhibition of P450s is far more effective than inhibition of phase II enzymes, and inhibitory potency increases with increasing chain length (*58, 59*). Studies carried out under the same conditions used in the rat NNK/PEITC lung tumor inhibition bioassays clearly demonstrate that prolonged selective inhibition of NNK metabolic activation in the rat lung is a major mechanism by which PEITC inhibits tumor formation (*67, 154, 200*). Similar inhibition of NNAL metabolic activation in the lung, but not the liver, is observed. Inhibition of reconversion of NNAL to NNK may also play a role in the inhibitory effect of PEITC on NNK tumorigenesis (*67*). Consistent with the inhibition of NNK metabolic activation in the lung by PEITC is its inhibition of hemoglobin adduct formation (Figure 10), an effect also observed in rats treated with PHITC (*155, 156*). In tandem with these effects, NNAL plus NNAL-Gluc excretion in the urine increases, consistent with inhibition of metabolic activation (*155, 156*). Increases in levels of NNAL plus NNAL-Gluc in the urine of smokers who consumed watercress as a source of PEITC have also been noted, consistent with the rat studies and the known ability of PEITC to inhibit P450 1A2, one of the P450s involved in human hepatic metabolism of NNK (Table 4) (*138, 165, 168*).

PEITC, a naturally occurring isothiocyanate with little toxicity at the doses required for chemoprevention of lung tumorigenesis by NNK, is presently the lead compound for chemoprevention of NNK carcinogenesis in smokers. Phase I clinical trials to determine its toxicity and pharmacokinetics in apparently healthy smokers are being initiated.

2.8. Other Biological Effects of NNK and NNAL. These are summarized in Table 16. Consistently, NNK

Table 16. Other Biological Effects of NNK[a]

Effect	Reference
Mutagenicity	*88, 89, 188, 327−339*
Other genotoxicity	*235, 327, 328, 330, 331, 338−346*
Toxicity and related effects	*206, 208, 212, 235, 240, 242, 331, 347*
Transformation	*136, 137, 331, 348−351*
Mitogenesis and other cellular effects	*183, 317, 352−372*
Immunosuppression	*309, 326, 373*

[a] Condensed version; full table appears in Supporting Information.

is mutagenic in *Salmonella typhimurium* TA 100 and TA 1535, in the presence of activating systems, generally S9 or microsomes from the livers of rats or hamsters pretreated with Aroclor or other P450 inducers. NNK and [*methylene*-D_2]NNK have similar mutagenicities, whereas [*methyl*-D_3]NNK is inactive in strains TA 100 and TA 1535. Moreover, two precursors to the pyridyloxobutylating species derived from NNK—NNKOAc and CNPB—are more mutagenic in TA 100 and TA 1535 than are the corresponding precursors to the methylating species—AMMN and carbethoxynitrosaminomethane (*183, 188*). These results demonstrate the importance of DNA pyridyloxobutylation in mutagenesis by NNK in *S. typhimurium*. These results do not necessarily correlate with the respective roles of DNA pyridyloxobutylation and methylation in NNK carcinogenesis.

A number of inhibitors of NNK mutagenesis have been identified. However, there is no clear relationship between inhibitors of mutagenicity and inhibitors of carcinogenicity. For example, D-limonene inhibits NNK tumorigenicity but not mutagenicity, whereas esculetin and (−)-esculin inhibit mutagenicity but not tumorigenicity (Tables 15 and 16). These differences result in part from the different models which have been used. Most studies on inhibition of tumorigenicity have been done in the A/J mouse lung tumor models, whereas many of the studies on inhibition of mutagenicity have been performed with hamster liver microsomes as the activating system.

Other genotoxic effects of NNK include induction of unscheduled DNA synthesis in rat hepatocytes, chromosome aberrations, sister chromatid exchanges, and micronuclei. An important role for detoxification by glucuronidation has been proposed as a protective mechanism against micronuclei formation (*346*).

NNK transforms cells to a neoplastic phenotype. This is observed in investigations with rat tracheal epithelial cells and human bronchial epithelial cells (*331, 349, 351*). In the latter, transformation is associated with increased expression of cyclin E (*351*). The dependence of transformation on activation of NNK by P450s is clearly shown by studies using C3H/10T1/2 cells expressing human P450 2A6 (*136, 137*).

NNK is mitogenic in various lung cancer cell lines and in hamster pulmonary neuroendocrine cells (*356−358*). The growth stimulation in cell lines with neuroendocrine or Clara cell features is inhibited by antagonists of nicotinic cholinergic receptors, suggesting a role for binding of NNK to this receptor. In a human lung adenocarcinoma cell line treated with NNK, stimulation of cell growth is inhibited by the β-adrenergic antagonist propanolol.

Hypermethylation of the estrogen receptor promoter is observed in 14−22% of mouse and rat lung tumors induced by NNK and in a similar percentage of lung tumors from smokers (*364*). In contrast, spontaneous lung tumors from A/J mice and plutonium-induced lung tumors from rats have a very high incidence of estrogen receptor methylation. Thus, gene-specific promoter methylation could be a biomarker associated with specific carcinogen exposures (*364*).

Overexpression of *myc* and a decrease in *Rb* transcripts have been noted in hamster lung after treatment with NNK (*361*). Increases in expression of *myc*, *raf*, and *Hras* are also observed in mouse lung after NNK administration (*363*).

NNK is immunosuppressive when administered chronically to A/J mice (*326*). The ability of various nonsteroidal antiinflammatory drugs such as sulindac to inhibit NNK-induced lung tumorigenesis is related to their inhibition of NNK-induced immunosuppression suggesting that their chemopreventive effects may be mediated through modulation of prostaglandin E_2 synthesis (*326*).

3. NNN

3.1. NNN Metabolism. NNN metabolism pathways are illustrated in Figure 12. Three types of reactions have been observed: pyridine N-oxidation, hydroxylation of the pyrrolidine ring (including α-hydroxylation at the 2′- and 5′-positions and β-hydroxylation at the 3′- and 4′-positions), and norcotinine formation. These will be discussed in the following sections on in vitro and in vivo metabolism.

3.1.1. In Vitro Metabolism of NNN. Studies on the in vitro metabolism of NNN are summarized in Table 17. Other than liver, studies have focused on rat target tissues for NNN carcinogenicity—esophagus and nasal mucosa—as well as oral tissue which appears to be closely related to esophagus with respect to NNN metabolism.

3.1.1.1. Pyridine N-Oxidation. NNN *N*-oxide is a detoxification product of NNN metabolism. It is observed in rat liver in substantial quantities but only in limited amounts or not at all in extrahepatic rat tissues (*69, 74, 78, 79, 376, 378, 379*). It has not been reported as a metabolite of NNN in human liver but is observed in several other human tissues (*116, 118, 384−386*). The enzymology of NNN *N*-oxide formation has not been investigated.

3.1.1.2. α-Hydroxylation. α-Hydroxylation of NNN at the 2′-position produces an unstable intermediate—2′-hydroxyNNN—which spontaneously loses HONO yielding myosmine or ring-opens to produce 4-(3-pyridyl)-4-oxobutane 1-diazohydroxide (**6**), the same intermediate formed upon α-methyl hydroxylation of NNK (*191, 374*). The metabolites ultimately formed by this pathway are keto alcohol, diol, and keto acid; their sum indicates the extent of NNN 2′-hydroxylation (*74, 78, 106, 378, 379, 381*). This common pathway in NNK and NNN metabolism apparently leads to the same adducts with DNA and hemoglobin (*78, 180, 199, 247*). Adduct formation from NNN has been observed only by this pathway. Studies with stable precursors to 2′-hydroxyNNN and the related diazohydroxide **6** indicate that there is substantial mutagenic activity associated with this pathway and it is regarded as the major route of NNN metabolic activation (*192, 374*).

5′-Hydroxylation of NNN yields 5′-hydroxyNNN which spontaneously ring-opens to 1-(3-pyridyl)-4-oxobutane 1-diazohydroxide (**23**) (*192, 374*). This reacts with H_2O

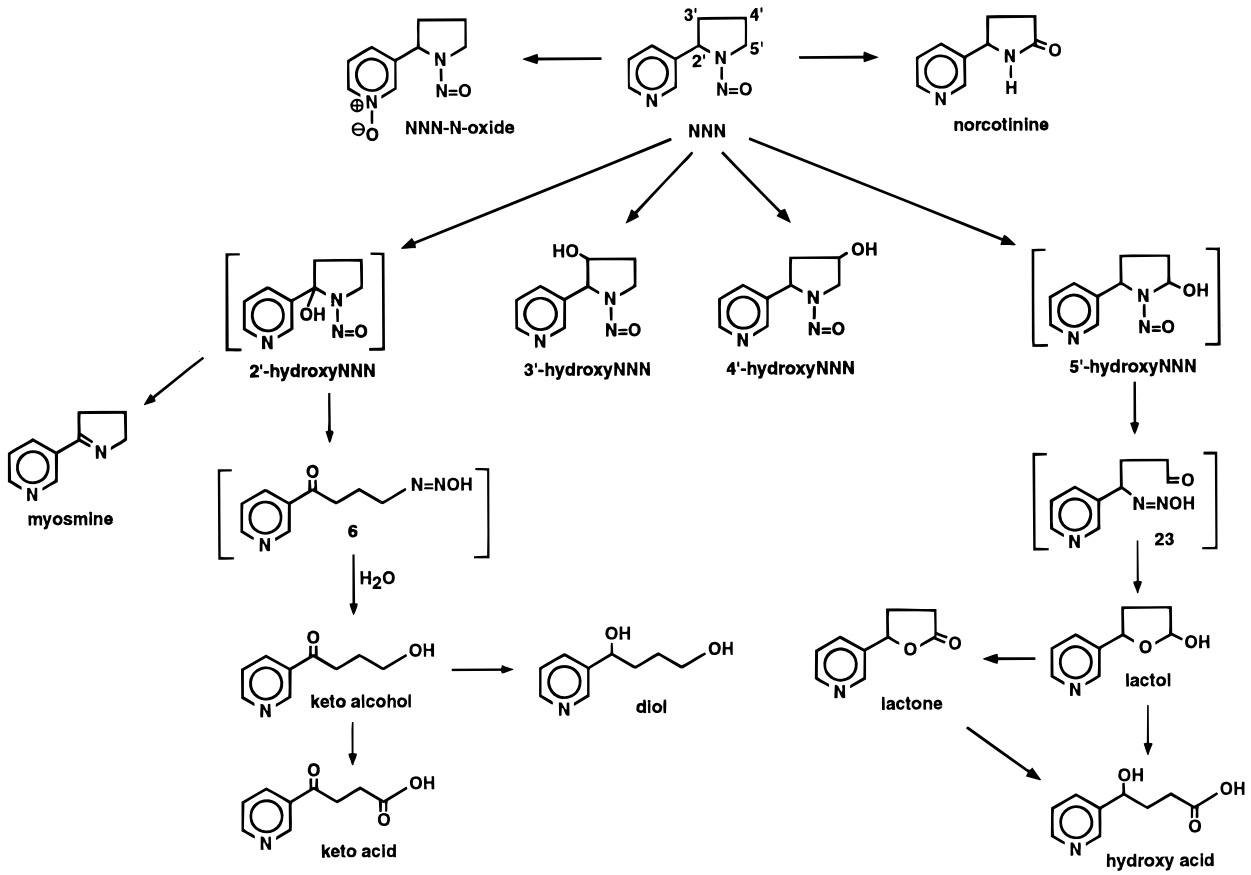

Figure 12. NNN metabolism pathways as determined by studies in laboratory animals.

Table 17. In Vitro Metabolism of NNN[a]

Species	Tissue	Conditions	Reference
Rat	Liver	Microsomes, tissue culture	*374−378, 381, 382*
	Esophagus	Microsomes, tissue culture	*78, 378−382*
	Nasal mucosa	Tissue culture	*74, 381*
	Oral tissue	Tissue culture	*78−80, 381*
	Lung	Cell culture	*69*
Hamster	Liver	Microsomes	*377*
	Esophagus	Tissue culture	*378*
Mouse	Lung	Tissue culture	*106*
Human	Liver	Microsomes	*118, 134, 384, 386*
	Esophagus	Tissue culture	*385*
	Colon	Tissue culture	*383*
	Various	Tissue culture	*116*

[a] Condensed version; full table appears in Supporting Information.

producing a hydroxy aldehyde that cyclizes to lactol. A precursor to 5′-hydroxyNNN, 5′-acetoxyNNN, is mutagenic in *S. typhimurium*, but a precursor to diazohydroxide **23** does not show mutagenicity (*192, 374*). Adducts from this pathway have not been identified but would be expected based on studies of *N*-nitrosopyrrolidine−DNA adduct formation (*201, 202*). Presently, it is not clear whether 5′-hydroxylation is an activation or detoxification pathway of NNN.

The 2′- and 5′-hydroxylation reactions in rat liver are dependent on NADPH, inhibited by CO, and specifically induced or repressed by pretreatment with MC, PB, and Aroclor (*374, 377, 382*). Similar results are reported in hamster liver (*377*). Most evidence is consistent with major P450 involvement in these reactions. Similarly, P450s appear to be the major catalysts of NNN α-hydroxylation in rat esophagus and nasal mucosa (*74, 382*). A P450 that efficiently metabolizes low concentrations of NNN is present in the rat esophagus and probably plays a major role in the metabolic activation and carcinogenicity of NNN in this tissue (*382*). There is no evidence for monoamine oxidase involvement, based on histochemical studies (*387*).

There is some consistency among ratios of 2′-hydroxylation to 5′-hydroxylation in different rodent tissues and their susceptibility to carcinogenesis by NNN. Thus, the 2′-hydroxylation-to-5′-hydroxylation ratio is typically 2−4 in rat esophagus and nasal mucosa, the main target tissues of NNN in the rat (*74, 78, 378, 382*). In liver, a nontarget tissue, 2′-hydroxylation-to-5′-hydroxylation ratios are 0.3−1.4 (*378, 381, 382*). Hamster esophagus, a nontarget tissue, predominantly 5′-hydroxylates NNN (*378*). Moreover, esophageal metabolism of the weak esophageal carcinogen NAB occurs mainly at the 6′-position (equivalent to 5′-hydroxylation of NNN) (*379*). These results are consistent with a role for 2′-hydroxylation in tumor induction by NNN and 5′-hydroxylation in detoxification. On the other hand, 5′-hydroxylation and 2′-hydroxylation occur to equal extents in hamster trachea, another NNN target tissue (*388*).

Human liver microsomes catalyze predominantly NNN 5′-hydroxylation (*118, 384, 386*). In one study, the ratio of 5′-hydroxylation to 2′-hydroxylation was 40 (*118*). Human liver microsomes metabolize NNN more rapidly than NNK, NNAL, or BaP (*118*). Convincing evidence for a major role of P450 2A6 in NNN 5′-hydroxylation in human liver has been presented, whereas P450 3A4 is involved in 2′-hydroxylation (*386*). P450 2A6 is also important in human hepatic 5′-hydroxylation of nicotine,

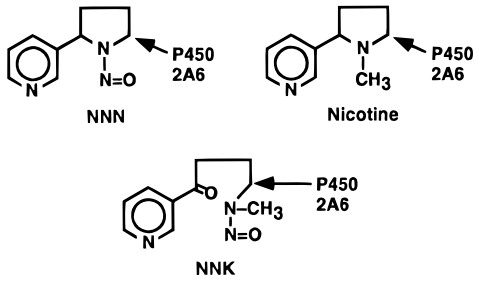

Figure 13. Structural similarities among NNN, nicotine, and NNK relating to their metabolism by P450 2A6, based on studies by Murphy, Patten, and others (*128*, *386*, *389*).

Table 18. In Vivo Metabolism of NNN[a]

Species	Reference
Rat	*145*, *171*, *374*, *376*, *379*, *391*, *392*
Hamster	*157*, *158*, *171*
Mouse	*158*, *171*, *393*−*395*
Pig	*396*
Monkey	*159*
Baboon	*158*

[a] Condensed version; full table appears in Supporting Information.

the first step in the metabolism of nicotine to its major metabolite cotinine (*389*). Moreover, P450 2A6 plays a role in human hepatic and pulmonary NNK α-methylene hydroxylation (*128*). These observations are consistent with the structural similarities of NNN, nicotine, and NNK as shown in Figure 13. 5′-Hydroxylation is observed in several human tissues other than liver, but 2′-hydroxylation less frequently (*116*, *385*).

3.1.1.3. β-Hydroxylation. This has been reported in one study and is clearly a minor pathway in rat hepatic microsomes (*376*).

3.1.1.4. Norcotinine. Norcotinine is observed in cultured mouse lung and, provisionally, in rat oral mucosa incubated with NNN (*78*, *106*). Studies on its origin in vitro have not been reported. It could result from denitrosation followed by oxidation of nornicotine or from oxidation of 5′-hydroxyNNN followed by denitrosation. Evidence for alcohol dehydrogenase-mediated metabolism of hydroxylated NNN has been suggested (*390*). Alternatively, norcotinine could be formed via P450-mediated radical formation at the 5′-position followed by loss of NO• giving *iso*-myosmine, followed by oxidation to norcotinine.

3.1.2. In Vivo Metabolism of NNN. Studies on NNN metabolism in vivo are summarized in Table 18. NNN is rapidly distributed and metabolized. Autoradiography demonstrates high initial uptake and tissue binding in the nose. The salivary glands and melanin-containing tissues are also frequent targets of NNN accumulation (*391*−*394*). NNN metabolites are excreted mainly in urine. Consistently, metabolites resulting from α-hydroxylation, pyridine N-oxidation, and denitrosation are observed in urine along with a small amount of unchanged NNN (*157*, *171*, *374*, *376*, *379*). No information is available on NNN or its metabolites in human urine.

3.1.1.1. Pyridine N-Oxidation. Urinary NNN *N*-oxide accounts for 7−11% of the dose in rats and 2.5% in hamsters (*157*, *171*). There are not major differences in excretion of NNN *N*-oxide among the rat, hamster, and mouse (*171*). It is not detected in minipig or marmoset urine (*159*, *396*). Excretion of NAB *N*-oxide in rat urine accounts for 30% of the NAB dose compared to 11% for NNN *N*-oxide (*379*). Since the *N*-oxides are detoxification products, this difference may account in part for the lower carcinogenicity of NAB than NNN. There is no evidence for further metabolism of NNN *N*-oxide (*171*).

3.1.2.2. α-Hydroxylation. Hydroxy acid and keto acid are the major urinary metabolites of NNN resulting from α-hydroxylation (*171*, *374*). Hydroxy acid results mainly from the 5′-hydroxylation pathway. It is formed via lactol and/or lactone which are minor urinary metabolites, or not detected (*171*, *374*). Hydroxy acid formation from keto acid is minimal, as it accounts for less than 1% of the dose of keto acid administered to rats (*171*). Moreover, keto acid is metabolized predominantly to (*R*)-hydroxy acid, while NNN is metabolized to a 1:1 mixture of (*R*)- and (*S*)-hydroxy acid (*172*). Keto acid is a product of 2′-hydroxylation via keto alcohol (*171*). In rats, hydroxy acid decreases with decreasing dose while keto acid increases suggesting the presence of a high-affinity enzyme for NNN 2′-hydroxylation in liver (*171*, *382*).

Hydroxy acid and keto acid are the major urinary metabolites of NNN in rodents, minipig, and marmoset (*157*, *159*, *171*, *374*, *396*). On the basis of in vitro studies, hydroxy acid is expected to be a major urinary metabolite of NNN in humans, but this has not been established.

3.1.2.3. Norcotinine. This metabolite is consistently observed in the urine of NNN-treated rats and has also been reported in minipig urine (*171*, *374*, *396*). It is not formed in significant quantities in the metabolism of nornicotine in the rat (*171*). The origin of norcotinine requires further study.

3.2. DNA Binding of NNN

3.2.1. In Vitro Studies. 2′- and 5′-Hydroxylation of NNN are the only metabolic pathways known to lead to DNA damage. 2′-Hydroxylation generates the same intermediate—4-(3-pyridyl)-4-oxobutane 1-diazohydroxide (**6**; Figures 2−4, 12)—as does methyl hydroxylation of NNK (*46*, *191*, *374*). The fate of this intermediate and its role in DNA binding are discussed in Section 2.2.1.1.2, and NNK DNA binding is outlined in Figures 2−4. Formation of HPB-releasing DNA adducts is therefore expected upon 2′-hydroxylation of NNN, and this has been observed as discussed further below. 2′-Hydroxylation of NNN could also yield an electrophilic nitrosiminium ion. Evidence for this pathway has been obtained in studies on 2′-acetoxyNNN, but its relationship to DNA adduct formation is not known (*191*). Little is known about the fate of diazohydroxide **23** from 5′-hydroxyNNN. 5′-AcetoxyNNN is mutagenic in *S. typhimurium* without activation, indicating that adducts are formed by this pathway (*374*). However, a stable precursor to diazohydroxide **23** is less mutagenic in *S. typhimurium* than CNPB, indicating that 5′-hydroxylation may be a less important pathway than 2′-hydroxylation (*192*). Diazohydroxide **23** from 5′-hydroxylation is structurally related to 4-oxobutane 1-diazohydroxide, formed upon α-hydroxylation of *N*-nitrosopyrrolidine. Adduct formation from this intermediate has been extensively characterized, and similar adducts may be formed upon 5′-hydroxylation of NNN (*201*, *202*, *397*−*399*).

HPB-releasing adducts are present in DNA of rat nasal mucosa and esophagus cultured with NNN, consistent with extensive 2′-hydroxylation in these tissues (*78*, *181*). In esophagus, HPB releasing DNA adducts are formed more extensively from NNN than from NNK, consistent with the higher carcinogenicity of NNN in this tissue (*78*). Several unidentified DNA adducts are observed in rat

Table 19. NNN Tumorigenicity[a]

Strain/Species	Route	Target Organs	Reference
F-344 rat	p.o.	Esophagus, nasal cavity	*381, 404, 412*
	s.c.	Nasal cavity	*17, 226, 381, 406*
	gavage	Esophagus	*279*
Sprague−Dawley rat	p.o.	Nasal cavity	*405*
BDVI rat	gavage	Predominantly nasal cavity	*407*
Chester Beatty mouse	i.p.	Lung	*7*
Swiss mouse	topical	None	*408*
A/J mouse	i.p.	Lung	*16, 106, 195, 283, 301*
CFLP mouse	topical	None	*409*
Sencar mouse	topical	None	*282*
Swiss or BALB/c mouse	oral	Lung, forestomach	*284, 311*
Syrian golden hamster	s.c.	Trachea	*157, 410*
	topical, check pouch	None	unpublished, *314, 318, 413, 414*
	i.p.	Trachea, nasal cavity	*411*
	p.o.	Trachea, nasal cavity	*412*
Mink	s.c.	Nasal cavity, forebrain	*321, 415*

[a] Condensed version; full table appears in Supporting Information.

nasal mucosa cultured with NNN including one that releases HPB upon acid hydrolysis, and putative dG adducts are reported in incubations of NNN, dG, and rat liver microsomes (*181, 400*).

3.2.2. In Vivo Studies. HPB-releasing adducts are present in acid or enzyme hydrolysates of hepatic DNA from NNN-treated rats and in acid hydrolysates of pulmonary DNA from NNN-treated mice (*180, 181, 195*). The properties of these adducts appear to be identical to those isolated from NNK-treated animals. Binding of NNN to DNA is observed in several rat tissues including nasal mucosa, lung, and liver, but adducts were not identified most likely due to the relative insensitivity of the radiometric methods employed (*180, 181, 392, 401*). The development of the GC/MS method for analysis of HPB-releasing adducts permits their detection in rat nasal mucosa after treatment with NNN (*199*). They are present in the respiratory and olfactory parts of the nasal mucosa, with the respiratory mucosa levels being higher. Consistent with expectations, O^6-mG is not detected in nasal mucosa or liver of rats treated with NNN (*178*).

HPB-releasing adducts are present in lung DNA from smokers (*198*). These adducts could result from metabolic activation of NNK, NNN, or both.

3.3. Protein and Tissue Binding of NNN. Incubation of NNN with cultured rat tissues leads to binding to nasal mucosa greater than liver and esophagus, on a per milligram of tissue basis (*391*). In vivo binding of NNN to nasal mucosal protein also exceeds that in liver (*392*). These results are consistent with the high activity of the rat nasal mucosa for NNN metabolic activation (*74*). Similar results are obtained in the marmoset (*159*). Protein binding has been used to characterize rat liver microsomal metabolism of NNN; the results indicate that NNN metabolic activation is catalyzed by P450s, which agrees with all other data (*402*). NNN also binds to hamster buccal pouch keratinocytes, human gingival fibroblasts, and Chang liver cells; binding is enhanced in keratinocytes by TPA pretreatment (*403*).

NNN forms hemoglobin adducts in rats (*247*). As expected, HPB-releasing adducts are formed, presumably via 2′-hydroxylation and diazohydroxide **6**, as in methyl hydroxylation of NNK. However, the amount of the HPB-releasing hemoglobin adducts formed from NNN is only about 16% as great as that from NNK. The presence of HPB-releasing adducts in hemoglobin of smokers and snuff-dippers, as discussed in section 2.3, could be due to 2′-hydroxylation of NNN and/or methyl hydroxylation of NNK.

3.4. Carcinogenicity of NNN

3.4.1. Rat. Carcinogenicity studies of NNN are summarized in Table 19. The esophagus and nasal mucosa are the main target tissues of NNN in the rat. Few other tumors are reproducibly observed. The relative proportions of esophageal and nasal tumors are strongly affected by the route of administration. In four studies in F-344 rats, administration of NNN in the drinking water or in a liquid diet produces esophageal and nasal tumors (*381, 404, 412*).[5] In four studies in F-344 rats, administration of NNN by sc injection causes either nasal tumors exclusively or predominantly, with some esophageal tumors (*17, 226, 381, 406*). NNN given by gavage induces mainly nasal tumors, similar to sc treatment (*407*). Both the esophagus and nasal mucosa can metabolically activate NNN, and DNA adducts are detected in both tissues. Autoradiographic studies in rats show accumulation and binding of NNN in both tissues after iv injection, but the levels are higher in the nasal mucosa than the esophagus (*391, 392*). More NNN may reach the esophagus upon administration in the drinking water, via direct contact, than after sc injection or gavage. This may explain the higher incidence of esophageal tumors in rats dosed with NNN in the drinking water, but this has not been established. One curious finding is the apparent lack of esophageal tumors in the Sprague−Dawley rat after po administration of NNN (*405*). This is not due to differences in esophageal metabolism of NNN in F-344 and Sprague−Dawley rats (*378*).

The esophageal tumors are mainly papillomas; squamous cell carcinomas are sometimes observed (*404, 412*). The papillomas can be lethal by blocking the esophagus. The malignant nasal tumors are frequently olfactory neuroblastomas; rhabdomyosarcomas are also observed (*17, 407*). The benign tumors arise from the respiratory epithelium. Squamous cell carcinomas of the nasal cavity are observed in rats treated with NNN *N*-oxide (*412*).

Only limited dose−response data are available. In a recently completed study, Stoner and Morse induced a 71% incidence of esophageal tumors with 5 ppm in the drinking water, which is about one-third of the previously reported lowest total dose of 1 mmol/rat (*381*).[5] The

[5] G. D. Stoner and M. A. Morse, unpublished results.

Table 20. Other Biological Effects of NNN[a]

Effect	Reference
Mutagenicity	*327−330, 334, 338, 374, 417, 418*
Other genotoxicity	*51, 237, 239, 241, 244, 327, 328, 340, 344, 345*
Viral interactions	*369−371*
Other cellular effects	*147, 347, 353, 354, 359, 360, 373, 419−428*

[a] Condensed version; full table appears in Supporting Information.

esophageal carcinogenicity of NNN may be greater than previously realized.

The lowest sc dose of NNN tested is 1 mmol/kg total, which induces approximately a 50% incidence of nasal tumors, mainly benign. The lowest gavage dose is approximately 0.8 mmol/kg, which induces about a 20% incidence of benign nasal tumors (*407*). NNN and NNK appear to have similar carcinogenic activities toward the rat nasal cavity (*199, 226*).

A mixture of NNN and NNK applied to the rat oral cavity produces a significant incidence of oral tumors and some lung tumors (*274*). NNK alone is ineffective in this regard, giving only lung tumors (*277*). NNN has not been tested by oral swabbing in the rat.

3.4.2. Mouse. NNN consistently induces lung tumors in the mouse, but the effect is far weaker than observed with NNK. The first bioassay of NNN was carried out in Chester Beatty mice (*7*). Other strains showing lung tumors upon treatment with NNN are A/J, Swiss, and BALB/c (Table 19). The A/J mouse results are reproducible; thus, a total dose of 100 μmol/mouse, administered over 7 weeks, induces 1−2 lung tumors/mouse (*16, 106, 283, 301*). A few salivary gland tumors have been observed, but they may not be treatment-related (*16, 416*).

Three studies demonstrate lack of mouse skin tumorigenicity of NNN (*282, 408, 409*).

3.4.3. Hamster. Four studies of NNN tumorigenicity in the hamster give consistent results, with tracheal and nasal tumors being observed, independent of the route of administration: sc, ip, or po (*157, 410−412*). The tracheal tumors are squamous papillomas (*411*). The nasal tumors arise from the olfactory epithelium and can be invasive (*411*). Tumor incidence is low at doses below 1 mmol/hamster administered ip or sc over 25 weeks (*157, 411*).

Tumors are not produced upon application of NNN to the cheek pouch (*314, 414*).[4]

3.4.4. Mink. The mink is the only nonrodent model in which tobacco-specific nitrosamines have been tested (*321, 415*). The expanded nasal mucosa of the mink may increase its susceptibility to nasal carcinogens. NNN is highly specific for nasal tumor induction in the mink which appears quite sensitive to its carcinogenic effects (*415*). The tumors are primarily esthesioneuroepitheliomas with invasion of the brain. NNK and a mixture of NNN and NNK are also carcinogenic in the mink, inducing mainly nasal tumors (*321*).

3.5. Other Biological Effects of NNN. NNN is mutagenic in *S. typhimurium* TA 100 and TA 1535 but not TA 98 or TA 1538 (Table 20). In this respect, it is similar to NNK but its mutagenic activity is less than that of NNK (*327*). Nicotine and cotinine have little effect on NNN mutagenicity which contrasts to the inhibition of NNN metabolic activation by nicotine in the rat oral tissue. In the mutagenicity study, the activating system is liver S9 prepared from Aroclor-treated rats, and the concentration of NNN is much higher than in the rat oral tissue studies (*79, 338*). Therefore, different P450 enzymes are probably involved in the activation of NNN in these systems.

NNN displays other genotoxic effects including induction of unscheduled DNA synthesis in rat hepatocytes, induction of micronuclei in mouse bone marrow, and induction of SSB. As in the mutagenicity experiments, the effects of NNN are generally less than those of NNK (*51, 237, 239, 327, 340*).

Studies using porcine oral mucosa demonstrate that NNN readily penetrates nonkeratinized tissues such as the floor of the mouth and buccal mucosa, and this penetration can be enhanced by ethanol (*421, 423*). These observations may be important with respect to the role of NNN as an oral carcinogen. Other studies suggest that NNN can change lipid synthesis and distribution in hamster oral cells (*428*).

4. NAB, NAT, NNA, *iso*-NNAL, and *iso*-NNAC

Two studies demonstrate that NAB is a relatively weak esophageal carcinogen in the rat, with activity significantly less than that of *N*-nitrosopiperidine or NNN (*8, 404*). NAB shows no activity in Syrian golden hamsters, while a similar dose of NNN produces a high incidence of tracheal tumors (*410*). NNN and NAB have similar activity for lung adenoma induction in the A/J mouse (*301*).

Approximately 25−30% of the NAB dose is excreted in the urine as NAB *N*-oxide, whereas only 6−11% of an equivalent dose of NNN is detected as NNN *N*-oxide in urine. Less than 10% of the NAB dose is detected in urine as products of α-hydroxylation, while the corresponding figure for NNN is 37%. These results suggest that hepatic pyridine N-oxidation is more facile for NAB than NNN, which may provide an efficient route for detoxification of NAB in the rat thus explaining its lower carcinogenicity than NNN (*379*). Differences are also noted in the esophageal metabolism of NAB and NNN. The 2′-hydroxylation-to-5′-hydroxylation ratio for NNN is 3, while the 2′-hydroxylation-to-6′-hydroxylation ratio for NAB is only 0.2−0.4 (*379*). DNA adduct formation by NAB has not been examined.

NAT shows no carcinogenic activity in one study in rats, in which total doses of 1, 3, and 9 mmol/kg were administered over 20 weeks by sc injection 3 times weekly (*226*). No metabolic studies have been reported. NAT inhibits the metabolism of NNK, but not NNN, in cultured rat oral tissue (*79*).

NNA is inactive as a lung tumorigen in the A/J mouse (*16*). *iso*-NNAL shows no tumorigenic activity when applied topically to Sencar mice or when administered by ip injection to A/J mice (*282, 301*). *iso*-NNAC is inactive in the hepatocyte primary culture DNA repair assay and as a tumorigen in the A/J mouse (*286, 301, 429*). Approximately 75% of *iso*-NNAC is excreted unchanged in the rat, mainly in the urine (*430, 431*). In this respect, *iso*-NNAC is similar to other nitrosamino acids. There is no evidence for endogenous formation of *iso*-NNAC in humans exposed to nicotine or cotinine (*431*).

5. Summary and Conclusions

The work reviewed here provides the basis for some attempted generalizations, although it is recognized that these could ultimately become obsolete or even laughable. Nevertheless, at least at the present time, there is little reason to doubt the following:

α-Hydroxylation is the major route of metabolic activation of NNK and NNAL. α-Hydroxylation activities are generally highest in rat target tissues of NNK carcinogenicity, as are the resulting DNA adducts. The formation of α-hydroxymethylNNK has been confirmed by isolation of its glucuronide conjugate. The α-hydroxylation reactions are catalyzed predominantly by P450s in rodents.

A variety of P450s are involved in NNK metabolic activation in rodents. These include members of the 1A, 2B, and 3A families. In humans, strong evidence indicates involvement of P450s 1A2, 2A6, and 3A4 in NNK activation.

NNK is rapidly converted to NNAL in rodents and humans. NNAL has similar carcinogenicity to NNK in rats and is somewhat less active in mice. It undergoes metabolic activation to DNA adducts by mechanisms similar to those observed for NNK. NNAL is conjugated as its glucuronide and excreted in the urine.

NNAL plus NNAL-Gluc are excreted in human urine and are good indicators of NNK dose in smokers and in nonsmokers exposed to environmental tobacco smoke. On the basis of levels of NNAL plus NNAL-Gluc in urine, the lifetime NNK dose in a smoker is not dissimilar from the lowest total NNK dose shown to induce lung tumors in rats. Levels of NNAL and NNAL-Gluc in urine are modified in rats and humans by dietary compounds such as PEITC and indole-3-carbinol.

Two types of DNA adducts—methylation and pyridyloxobutylation—are formed by α-hydroxylation of NNK and NNAL. The methylation adducts are 7-mG, O^6-mG, and O^4-mT. The pyridyloxobutylation adducts release HPB upon hydrolysis. Methylation and pyridyloxobutylation adducts are both involved to varying extents in carcinogenesis and mutagenesis by NNK.

NNK and NNAL form HPB-releasing hemoglobin adducts in rodents and humans. There is a predictable although complex relationship between levels of hemoglobin and DNA adducts in rats. Decreases in levels of hemoglobin adducts by treatment with chemopreventive agents such as PEITC parallel decreases in pulmonary DNA adducts and decreases in lung carcinogenicity in NNK-treated rats. Hemoglobin adducts of NNK have been quantified in smokers and snuff-dippers, but urinary metabolites of NNK may prove to be better biomarkers of NNK uptake and metabolism in humans.

NNK is the strongest carcinogen among the tobacco-specific nitrosamines in rodents. Its main target is the lung, where it induces predominantly adenomas and adenocarcinomas independent of the route of administration. The induction of lung tumors by NNK in F-344 rats and A/J mice obeys predictable dose−response relationships and is reproducible.

F-344 rat lung tumors result from efficient metabolism of low doses of NNK by lung cell types and persistence of the resulting DNA adducts. Pyridyloxobutylation of type II cell DNA and O^6-mG formation in Clara cell DNA appear to be critical for lung tumor induction. Formation and persistence of O^6-mG is critical for lung tumorigenicity in the A/J mouse.

GGT → GAT mutations in codon 12 of the *Kras* gene are observed in a high percentage of NNK-induced A/J mouse lung tumors, consistent with the role of O^6-mG. However, this is not the case in the rat, where *Kras* mutations have not been detected. The relationship of these findings to *KRAS* mutations observed in human lung tumors is unclear.

Many inhibitors of NNK-induced lung tumorigenesis have been identified, mainly by studies in the A/J mouse. Isothiocyanates are the most potent inhibitors among compounds administered before or during NNK treatment. Among these, the naturally occurring PEITC is the lead compound for chemoprevention in humans.

The major routes of NNN metabolic activation in rodents are 2′- and 5′-hydroxylation. The 2′-hydroxylation pathway predominates in rat target tissues of NNN: esophagus and nasal mucosa. The rat esophageal epithelium has a high-affinity enzyme, probably a P450, that 2′-hydroxylates NNN, leading to the formation of HPB-releasing adducts. These adducts are also present in the nasal mucosa of NNN-treated rats.

P450s are the major enzymes involved in NNN metabolic activation. In humans, P450 2A6 is important in 5′-hydroxylation and P450 3A4 in 2′-hydroxylation. P450 2E1 may also play some role.

NNN induces esophageal and nasal tumors in rats. The esophagus is the main target of NNN administered in the drinking water, while the nasal mucosa is the primary target when NNN is injected or gavaged. NNN consistently induces respiratory tract tumors in hamsters and mice.

A mixture of NNN and NNK swabbed in the rat oral cavity causes oral tumors, but NNK alone causes only lung tumors by this route. Neither NNN nor NNK induces oral tumors when applied to the hamster cheek pouch.

NNK, NNAL, and NNN are the most carcinogenic of the tobacco-specific nitrosamines that have been identified in tobacco products. NAB is a weak esophageal carcinogen. NAT, *iso*-NNAL, and *iso*-NNAC have not shown any activity.

The following areas are only some of those that require further research:

The stereochemical aspects of NNK and NNAL metabolism are incompletely understood, as are the pharmacokinetics of NNK and NNAL in rodents and humans. The role in NNK carcinogenesis of reconversion of NNAL to NNK is unclear.

Although multiple P450s are clearly involved in NNK metabolic activation, the specific P450s which mediate these reactions in the rodent and human lung have not been identified. The respective roles of Clara and type II cells in rat lung carcinogenesis by NNK require further definition.

The structures of the NNK− and NNN−DNA adduct(s) which release HPB upon hydrolysis have not been determined.

NNK pyridyloxobutylation inhibits AGT. The role of this process in NNK carcinogenicity and the relevant mechanisms have not been fully elucidated.

In vitro studies indicate that NNK metabolic activation in human lung occurs less extensively than in rodent or monkey lung. It is unclear whether this is due to disease state, tissue artifacts, or other factors. Further studies

on human pulmonary metabolism of NNK, and on NNK metabolism in vivo in humans, are needed.

Glucuronidation is important in NNK metabolism, but the enzymology and relevance of particular glucuronide metabolites have not been investigated in detail.

NNK causes immunosuppression in mice. Further studies on the mechanisms of this effect and its relevance to human carcinogenesis are required.

NNK, through a receptor-binding mechanism, is mitogenic in certain human lung cancer cell lines and in hamster pulmonary neuroendocrine cells. The mechanism of this effect and its role in carcinogenesis have not been fully elucidated.

NNN has a chiral center at the 2′-position, but the stereochemical aspects of NNN carcinogenicity and metabolism have not been studied.

The specific P450 that activates NNN and other nitrosamines in the rat esophagus has not been identified. It will be important to determine whether this P450 is expressed and active in human esophagus and oral tissue.

DNA adduct formation by 5′-hydroxylation of NNN is likely, but this pathway is largely uncharacterized.

Metabolic pathways of NAT and NAB are only poorly characterized, and the reasons for their low carcinogenic activities are mainly unknown.

The molecular consequences of DNA adduct formation by tobacco-specific nitrosamines are only partly understood. Fairly extensive work has been carried out on the *ras* pathway, but other studies relating specific types of DNA damage to mutagenesis and gene activation or inactivation are scattered, and a sequence of events connecting DNA damage with tumor formation has not been defined (as is the case for most carcinogens).

Although NNN and NNK are clearly major carcinogens in moist snuff, the mechanisms by which moist snuff induces oral cancer in humans require further investigation. There may be cofactors in this process that are unidentified. Moreover, the high exposure to NNK in snuff users suggests that they should be at risk for lung cancer, but this has not been explored.

Following lectures on this subject, there are seven questions which commonly are asked about tobacco-specific nitrosamines:

1. Is it NNK or BaP that causes lung cancer in smokers? Both are important. First, BaP is just one of a variety of PAH carcinogens in tobacco smoke including benzofluoranthenes, methylchrysenes, dibenz[*a*,*h*]anthracene, and others (*19*). Two of these, 5-methylchrysene and dibenz[*a*,*h*]anthracene, are more potent lung tumorigens than BaP in the A/J mouse model, although their concentrations in smoke are less than that of BaP (*19, 432*). BaP is an effective pulmonary carcinogen, inducing predominantly squamous cell carcinoma upon intratracheal instillation or implantation in the lung of rats or hamsters (*433, 434*). Lung tumor induction in these models depends on local application. BaP is slowly absorbed by the tracheal epithelium and extensively metabolized there, which partially explains its local carcinogenic effects (*435*). BaP−DNA adducts have been detected in human lung, and there is evidence that mutations in the *p53* gene could be due to these adducts (*436*−*438*). Collectively, these data support the role of BaP and other PAH as causative agents for squamous cell carcinoma of the lung in smokers. In the A/J mouse, BaP is somewhat more active than NNK as a lung tumorigen when given by gavage, but NNK is more potent when administered ip (*303*). As discussed, NNK induces adenocarcinoma of the lung in rats and hamsters, independent of the route of administration. HPB-releasing DNA adducts and 7-mG are higher in the lungs of smokers than nonsmokers, and both G−T and G−A mutations are prevalent mutations in the *p53* gene in adenocarcinoma of the lung (*437*). These results are consistent with a role for NNK in the induction of adenocarcinoma. Levels of NNK in cigarette smoke rose from 1978 to 1992, while levels of BaP decreased from 1960 to 1980 (*439*). These changes in NNK and BaP levels may partially explain the dramatic shift from squamous cell carcinoma to adenocarcinoma of the lung that has been observed in smokers (*28, 439, 440*). Thus, the available data indicate an important role for NNK in the etiology of adenocarcinoma of the lung in smokers.

2. Which P450 is involved in NNK metabolism? Multiple P450s are involved in NNK metabolism (Tables 3 and 4). In rodents, these include members of the 1A, 2B, and 3A families. In humans, strong evidence indicates the involvement of P450s 1A2, 2A6, and 3A4 in NNK activation.

3. Are people who use the nicotine patch exposed to tobacco-specific nitrosamines? There is no published evidence for the presence of tobacco-specific nitrosamines in the nicotine patch. Human exposure would depend on endogenous formation of the nitrosamines. This can occur in rats (*441*) and under the right conditions could take place in people who use the patch, but this has not been demonstrated.

4. Are NNK and NNN metabolites of nicotine? No. They could be formed endogenously by reaction of nitrite or related species with the appropriate precursors. There is no evidence that NNK is formed endogenously from nicotine. However, NNN is produced when nitrite is present, probably via metabolic conversion of nicotine to nornicotine, followed by nitrosation (*441*). Still, NNN would not be considered a “nicotine metabolite”.

5. Are tobacco-specific nitrosamines formed endogenously? See questions 3 and 4.

6. Are tobacco-specific nitrosamines found in products other than tobacco products? Other than the detection of tobacco-specific nitrosamines in nicotine chewing gum (*442*), there is no published evidence for this. The specificity of tobacco-specific nitrosamines for tobacco products is important when considering the exposure of nonsmokers to carcinogens in environmental tobacco smoke. Detection of NNAL or NNAL-Gluc in the urine of these individuals specifically implicates tobacco smoke as the source (*163, 443*).

7. Could one add PEITC to cigarettes to inhibit lung cancer? This is unlikely to work, because of the reactivity of PEITC. The heat generated in smoking and the stream of other constituents would probably result in very low delivery of PEITC to the lung. Other routes of administration of PEITC to smokers are being investigated.

In summary, there is now a large amount of data available on the biochemistry, carcinogenicity, and other biological properties of tobacco-specific nitrosamines, particularly NNN and NNK. How can one use this information? First, the tobacco specificity of these carcinogens is important as they provide a link between nicotine addiction and cancer that can be attributed only to tobacco, in contrast to other smoke carcinogens such

as PAH which occur in the diet and the general environment. As an example, the detection of NNAL in the urine of nonsmokers exposed to environmental tobacco smoke specifically implicates tobacco smoke as the source of this carcinogenic exposure, thereby strengthening the argument that passive smoking causes lung cancer (*163*, *443*). Second, by delineating mechanisms of tobacco-specific nitrosamine metabolic activation and detoxification in animal models, we can understand more fully their fate in humans. This is presently a major focus of our research, with the goal of elucidating factors which influence susceptibility to cancer development upon exposure to carcinogens. Differences in carcinogen metabolic activation and detoxification arguably can influence susceptibility, and such differences can be probed through genotyping and phenotyping approaches (*444*). Given the complexity of carcinogen metabolism, as exemplified here for NNK, we believe that carcinogen metabolite phenotyping will ultimately be a more useful approach than genotyping for identifying individual differences in carcinogen activation and detoxification. Third, by understanding mechanisms of cancer induction by tobacco-specific nitrosamines, rational approaches toward chemoprevention of tobacco-related cancers can be designed. This field has advanced remarkably in the past decade, and further significant progress is likely. As lung cancer therapy is still largely unsuccessful, chemoprevention remains as a viable way to prevent cancer death in addicted smokers and in ex-smokers. Finally, mechanistic studies on tobacco-specific nitrosamine-induced cancer continue to provide new insights on tobacco-related toxicology and carcinogenesis. One example is the recently demonstrated immunosuppressive activity of NNK, and a second is the detection of NNK in cervical mucus of smokers (*326*, *445*). Thus, the research described here will increase our understanding of mechanisms of cancer induction in humans and hopefully contribute to its prevention.

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Supporting Information Available: Full versions of Tables 1, 5−7, 9, 10, 13, and 16−20 are available in the reprints of this paper or through the ACS web site http://pubs.acs.org (28 pages). Further ordering information is given on any current masthead page.

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