Context matters: Contribution of specific DNA adducts to the genotoxic properties of the tobacco specific nitrosamine NNK
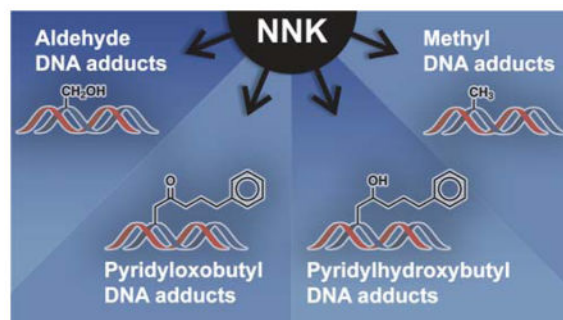
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Abstract

The tobacco specific nitrosamine, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) is a potent pulmonary carcinogen in laboratory animals. It is classified as a Group 1 human carcinogen by the International Agency for Cancer Research. NNK is bioactivated upon cytochrome P450 catalyzed hydroxylation of the carbon atoms adjacent to the nitrosamino group to both methylating and pyridyloxobutylating agents. Both pathways generate a spectrum of DNA damage that contributes to the overall mutagenic and toxic properties of this compound. NNK is also reduced to form 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL) which is also carcinogenic. Like NNK, NNAL requires metabolic activation to DNA alkylating agents. Methyl hydroxylation of NNAL generates pyridylhydroxybutyl DNA adducts and methylene hydroxylation leads to DNA methyl adducts. The consequence of this complex metabolism is that NNK generates a vast spectrum of DNA damage, any of which can contribute to the overall carcinogenic properties of this potent pulmonary carcinogen. This article reviews the chemistry and the genotoxic properties of the collection of DNA adducts formed from NNK. In addition, it provides evidence that multiple adducts contribute to the overall carcinogenic properties of this chemical. Which adduct contributes to the genotoxic effects of NNK depends on the context, such as the relative amounts of each DNA alkylating pathway occurring in the model system, the levels and genetic variants of key repair enzymes and the gene targeted for mutation.

Graphical abstract

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1. Introduction

Lung cancer is the leading cause of cancer mortality in the United States as well as globally.[1, 2] While the majority of all lung cancer cases are attributed to tobacco use, only a fraction of smokers get lung cancer, indicating that lung cancer risk is driven by gene-environment interactions.[3–6] While smoking cessation is an effective method of lung cancer prevention, many smokers find it difficult to quit due to their nicotine addiction. Therefore, identification of risk variants may assist in the identification of high risk individuals who then can be targeted for screening and/or prevention.

Tobacco smoke is a toxic mixture of over 5000 chemicals, including over 70 established carcinogens that have "sufficient evidence for carcinogenicity in laboratory animals or humans" according to the International Agency for Research on Cancer.[7, 8] Sixteen of these chemicals are classified as Group 1 human carcinogens.[8] Many of these chemicals require metabolic activation to be converted to electrophiles that bind covalently to DNA. The resulting DNA adducts are critical for tumor formation. If the DNA adducts persist and escape DNA repair processes, they will cause miscoding during DNA replication, leading to permanent mutations in critical growth control genes such as *ras* and *p53*. This results in genomic instability, loss of normal cellular growth control mechanisms and ultimately lung cancer.

The focus of this perspective is the tobacco chemical, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK). Formed from nicotine during the curing process of tobacco, NNK is a potent lung carcinogen in laboratory animals; it also induces liver, nasal and pancreatic tumors.[9–11] This compound induces lung adenocarcinomas in rodents at doses that are comparable to those experienced by smokers.[12] This type of cancer is now the most common type of lung cancer observed in humans, having surpassed squamous cell carcinoma. This shift in histology has not been attributed to improvements in diagnoses but rather to the changes in cigarette design, which increases the exposure of humans to tobacco-specific nitrosamines.[13] NNK metabolites have been detected in urine of smokers and individuals exposed to second hand smoke, indicating that humans are exposed to and metabolize this carcinogen.[14–17] Elevated levels of NNK metabolites in human urine are associated with increased lung cancer risk. [18–20] All these data support the classification of NNK as a human carcinogen.[21]

2. DNA reactive metabolites of NNK

NNK requires metabolic activation to DNA reactive metabolites to exert its tumorigenic effects.[22] Cytochrome P450 enzymes catalyze the oxidation of the α-carbon adjacent to the nitrosamine leading to the formation of unstable α-hydroxy metabolites that break down into DNA reactive metabolites (Figure 1). Since NNK is an unsymmetrical nitrosamine, it has two different α-hydroxylation pathways (Figure 1).[23–27] Methyl hydroxylation of NNK

generates a metabolite that decomposes to 4-(3-pyridyl)-4-oxobutanediazohydroxide and formaldehyde. The alkanediazohydroxide proceeds to 4-(3-pyridyl)-4-oxobutanediazonium ion which reacts with DNA bases to generate pyridyloxobutyl DNA adducts. Methylene hydroxylation of NNK leads to the formation of methanediazohydroxide and an aldehyde (4-oxo-1-(3-pyridyl)-1-butanone, OPB). The resulting methanediazonium ion reacts with DNA to generate methyl DNA adducts.

A major metabolic pathway of NNK is the reduction of the carbonyl group to form 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL) as a mixture of (R)- and (S)-enantiomers.[12] This is not a detoxification reaction because NNAL is carcinogenic.[12, 28] Like NNK, NNAL requires metabolic activation to DNA alkylating agents (Figure 1). Methyl hydroxylation of NNAL generates pyridylhydroxybutyl DNA adducts and methylene hydroxylation leads to DNA methyl adducts.[28–30]

The consequence of this complex metabolism is that NNK generates a vast spectrum of DNA damage, any one of which can contribute to the overall carcinogenic properties of this potent pulmonary carcinogen. The next section reviews the chemistry and toxicological effects of the multitude of adducts derived from NNK.

3. Chemistry and toxicology of NNK-derived DNA damage

3.1. Methyl DNA adducts

Methylene hydroxylation of NNK and NNAL generates 7-methyl-2′-deoxyguanosine (7-mdGuo) and O^6-methyl-2′-deoxyguanosine (O^6-mdGuo) (Figure 2) as well as other methyl DNA damage.[12] While not generally measured in DNA from NNK-treated animals, it can be assumed that the methanediazonium ion generated from NNK produces the same spectrum of methyl adducts as other methylating nitrosamines. The expected order of formation of adducts is 7-methylguanine > methyl phosphotriesters > O^6-methylguanine > 3-methyladenine > 1-methyl adenine $\cong$ 7-methyladeninine $\cong$ 3-methylcytosine $\cong$ 3-methylguanine > 3-methylthymidine $\cong$ O^2-methylthymine > O^4-methylthymine $\cong$ O^4-methylcytosine.[31, 32] While formamidopyrimidine (fapy) methyl adducts have been observed in animals treated with methylating nitrosamines,[31] this adduct has not been measured in DNA from NNK-treated animals.

3.1.1. Genotoxic properties of O^6-mdGuo—The major mutagenic and toxic methyl adduct is O^6-mdGuo.[33] It induces primarily GC to AT transition mutations.[34, 35] This adduct is predominantly repaired by O^6-alkylguanine DNA alkyltransferase (AGT). This protein facilitates the transfer of the methyl group from the O^6-position of guanine to a cysteinyl residue at the active site.[33] This transfer reaction renders AGT inactive and it is degraded.[36, 37] Therefore, constitutive levels of AGT determine the initial repair capacity of a cell. Mismatch repair (MMR) plays a major role in the toxic effects of O^6-mdGuo.[38–42] During the replication of this adduct, polymerases preferentially place T opposite the modified G. The MMR machinery recognizes this as a mismatch and removes the T. If the adduct is not repaired, the mismatch process enters a futile cycle since DNA replication will result again in a mismatch. This futile cycle can trigger apoptosis.[32, 43, 44]

3.1.2. Genotoxic properties of 7-mdGuo

While 7-mdGuo is the dominant adduct, it neither miscodes nor blocks replication.[44–46] The ring-opened methyl fapy adduct is a block to replication and can be mutagenic, depending on the sequence context.[46–50] Studies with site-specifically incorporated methyl FAPY adducts in primate cells indicated that this adduct induced base substitutions (primarily T but also A and C) and single and dinucleotide deletions.[50] Depurination of 7-mdGuo leaves behind an abasic site which is mutagenic and toxic if not repaired.[44] It is rapidly repaired by base excision repair (BER).

3.1.3. Genotoxic properties of minor methyl DNA adducts

There is evidence that the minor methyl adducts could contribute to the genotoxic effects of the methylating pathway. For example, O^4-methyl-2′-deoxythymidine (O^4-mdThd) induces primarily TA to CG and AT to TA mutations.[34, 35] While studies with site specifically incorporated adducts indicated that O^4-mdThd is more mutagenic than O^6-mdGuo, O^6-mdGuo is a more abundant adduct.[34, 35] 3-Methyl-2′-deoxyadenosine blocks replication, leading to cytotoxicity.[32, 44] There is also evidence that this adduct is weakly mutagenic.[32] Finally, O^2-methyl-2′-deoxythymidine (O^2-mdThd) is mutagenic inducing TA to GC and TA to AT mutations in *E. coli* and TA to AT mutations in human cells.[51, 52] This adduct also blocks DNA replication.[51, 52]

3.2. Pyridyloxobutyl DNA adducts

The chemical characterization of pyridyloxobutyl DNA damage was challenging since a majority of these adducts dealkylate, releasing 4-hydroxy-1-(3-pyridyl)-1-butanone (HPB) under strong acid or neutral thermal DNA hydrolysis conditions (Figure 1).[12, 53] The first chemically characterized pyridyloxobutyl DNA adduct was O^6-[4-3-(pyridyl)-4-oxobut-1-yl]-2′-deoxyguanosine (O^6-pobdGuo) (Figure 2).[54] Subsequent studies led to the characterization of three additional pyridyloxobutyl DNA adducts: 7-[4-3-(pyridyl)-4-oxobut-1-yl]-2′-deoxyguanosine (7-pobdGuo), O^2-[4-3-(pyridyl)-4-oxobut-1-yl]-2′-deoxycytosine (O^2-pobdCyd), and O^2-[4-3-(pyridyl)-4-oxobut-1-yl]-2′-deoxythymidine (O^2-pobdThd) (Figure 2).[55,56] Standards for N^2-[4-3-(pyridyl)-4-oxobut-1-yl]-2′-deoxyguanosine were prepared but this adduct was not observed in pyridyloxobutylated DNA.[57] The presence of phosphate adducts in pyridyloxobutylated DNA has been recently reported.[58] These adducts were detected as phosphotriesters (PTEs), with two nucleosides linked together by an alkylated phosphate group; 30 of the possible 32 PTEs were detected.

The pyridyloxobutyl DNA adducts have varying stability (Table 1). The most stable adducts are O^6-pobdGuo, O^2-pobdThd and the phosphate adducts.[54, 56, 58] O^6-pobdGuo and O^2-pobdThd will depurinate/depyrimidate when heated in 0.1 N HCl.[54, 56] The other pyridyloxobutyl DNA adducts, 7-pobdGuo and O^2-pobdCyd, undergo one of two reactions under neutral thermal hydrolysis conditions: they lose the sugar moiety to generate the nucleobase adducts, 7-[4-3-(pyridyl)-4-oxobut-1-yl]guanine (7-pobGua) and O^2-[4-3-(pyridyl)-4-oxobut-1-yl]cytosine (O^2-pobCyt) or they dealkylate to release HPB.[55, 56] The dealkylation reaction increases in strong acid hydrolysis conditions. Another possible source of HPB-releasing DNA adducts are 2-(3-pyridyl)-2,3,4,5-tetrahydrofuran adducts. Studies with model pyridyloxobutylating agents provide evidence for a reactive cyclic oxonium ion in the alkylation of nucleophiles by the pyridylxobutylating intermediate.[59] This cyclic

oxonium ion was trapped with nucleophiles such as thiols. While there is no direct evidence for these adducts in DNA, they are expected to be unstable and release HPB under all DNA hydrolysis conditions.[12, 53]

The relative levels of pyridyloxobutyl DNA adducts were determined in calf thymus DNA alkylated with the model pyridyloxobutylating agent, 4-(acetoxymethylnitrosamino)-1-(3-pyridyl)-1-butanone (NNKOAc); in the presence of esterase, NNKOAc generates the same pyridyloxobutylating agent formed following the oxidation of NNK by cytochrome P450 (Figure 3).[59] Quantitation of all these adducts in NNKOAc-treated DNA indicates that HPB-releasing adducts represent about 65% of the total pyridyloxobutyl DNA adducts.[60] The four characterized adducts account for the remaining amount. The relative levels of the four adducts is 7-pobdGuo > O^6-pobdGuo > O^2-pobdThd O^2-pobdCyd.[61] The phosphate adducts represented about 5% of the total DNA adducts in NNKOAc-treated calf thymus DNA.[58]

Indirect evidence for the formation of fapy adducts in DNA from NNKOAc-treated human cells was obtained using the comet assay in the presence and absence of formamidopyrimidine glycosylase.[62] Since more breaks were observed in the presence of the glycolyase, it was concluded that pyridyloxobutyl fapy adducts were present in the alkylated DNA. It is not known if pyridyloxobutyl fapy adducts are repaired as efficiently as methyl fapy adducts.

3.2.1. Genotoxic properties of O^6-pobdGuo

Several of the pyridyloxobutyl DNA adducts are mutagenic. O^6-pobdGuo is mutagenic, generating exclusively GC to AT mutations in bacteria and predominantly GC to AT mutations with some GC to TA transversion mutations in human cells.[63] It is repaired by AGT.[54, 64, 65] The bulky pyridyloxobutyl group gets transferred to the protein's active site, inactivating AGT.[65, 66] AGT is able to modulate the mutagenic properties of DNA pyridyloxobutylating agents at GC base pairs in both mammalian and bacterial systems, supporting the mutagenic properties of O^6-pobdGuo.[67, 68] Unlike other bulky O^6-alkyguanine adducts, O^6-pobdGuo is not a substrate for nucleotide excision repair (NER).[68] Studies with site-specifically incorporated O^6-pobdGuo indicated that this adduct was also not repaired by bacterial AlkD glycosylase or human alkyladenine glycosylase.[69]

3.2.2. Genotoxic properties of O^6-pobdThd

Another mutagenic pyridyloxobutyl DNA adduct is O^2-pobdThd. The major mutation induced by this adduct is TA to AT mutations with small amounts of TA to GC and/or TA to CG mutations in human embryonic kidney 293T (HEK293T) cells.[52] TA to GC mutations were the major mutation (37%) compared to TA to AT (12%) induced by this adduct in bacterial strains.[51] The removal of O^2-pobdThd from DNA was slower in Chinese hamster ovary (CHO) cells lacking the ERCC2 (XPD) gene (UV5 cells);[68] this is an important protein in the NER pathway. Therefore, O^2-pobdThd is likely a substrate for NER repair. Knockout of this repair pathway was also associated with increased NNKOAc-derived cytotoxicity in UV5 cells relative to the control cell line.[68] Consistent with these observation, this adduct is a block to replication in *E. coli* and HEK293T cells.[51, 52] The absence of NER in the UV5 CHO cell line also resulted in an increase in NNKOAc-induced *hprt* mutations, specifically TA to AT

mutations, providing support for the importance of O^2-pobdThd in the overall mutagenic activity of pyridyloxobutylating agents.[68] Pol ζ and other Y-family polymerases are thought to play a major role in the error-prone and error-free bypass of this bulky lesion.[52] This adduct is not a substrate for AGT, bacterial AlkD glycosylase or human Aag.[68, 69]

3.2.3. Genotoxic properties of 7-pobdGuo—The miscoding properties of 7-pobdGuo have not been investigated. A number of 7-alkylguanine adducts undergo imidazole ring-opening to mutagenic fapy adducts. Examples include fapy-7-methylguanine and aflatoxin B1-fapy adducts which both trigger GC to TA mutations.[70–72] It is not known if 7-pobdGuo forms a fapy adduct, but it is worth noting that pyridyloxobutylated DNA contains fapy glycosylase-sensitive adducts.[62] Since 7-pobdGuo spontaneously depurinates from DNA generating abasic sites,[55, 60] it is possible that this adduct contributes to the genotoxic properties of the pyridyloxobutyl pathway by generating abasic sites. There does not appear to be active repair of this adduct in mammalian cells.[68, 73]

3.2.4. Genotoxic properties of O^2-pobdCyd—The miscoding properties of O^2-pobdCyd have also not been studied. This adduct spontaneously depyrimidates from DNA, generating abasic sites.[56] While there is no evidence of active repair in mammalian cell lines,[68] the levels of this adduct are, in general, 10 times lower relative to the other pyridyloxobutyl DNA adducts in vivo than what is reported in *in vitro* alkylated DNA.[73] Therefore, active repair of this adduct may occur in vivo.

3.2.5. Genotoxic properties of abasic sites—It is possible that abasic sites could contribute to the overall genotoxic properties of NNK since there are multiple adducts that spontaneously decompose leaving behind abasic sites (see above). Abasic sites within GC base pairs cause GC to AT and GC to TA mutations.[74] Consistent with this hypothesis, the cytotoxic and mutagenic effects of NNKOAc were roughly doubled in CHO cell lines lacking functional XRCC1 (EM9 cells).[68] XRCC1 is an important scaffold protein that participates in numerous steps in the base excision repair (BER) pathway.[75] Therefore, the pyridyloxobutyl pathway generates DNA damage that requires XRCC1 activity for the cell to escape the cytotoxic and mutagenic effects of this damage. There may be specific pyridyloxobutyl DNA adducts that are substrates for BER glycosylases that initiate the BER process. Alternatively, pyridyloxobutylation of DNA generates abasic sites since both 7-pobdGuo and O^2-pobdCyd spontaneously depurinate/depyrimidate from DNA. These resultant abasic sites are expected to be repaired by the BER machinery. Since there was an increase in the mutation frequency of NNKOAc-induced AT to TA mutations in EM9 cells, abasic sites may be forming in AT base pairs. Studies with site-specific abasic sites indicate that they cause more AT to TA mutations than AT to GC mutations.[74] Therefore, abasic sites generated either by glycosylase catalyzed removal of pyridyloxobutylated adducts or by spontaneous depurination/depyrimidation may contribute to the mutagenic activity of pyridyloxobuylating agents.

3.3. Aldehyde DNA adducts

The aldehydes formed in NNK or NNAL metabolism are also capable of reacting with DNA. Formaldehyde reacts with the exocyclic amines of dAdo, dCyd and dGuo to form

reversible hydroxymethyl adducts, N^6-hydroxymethyl-2′-deoxyadenosine, N^4-hydroxy-2′-deoxycytotidine and N^2-hydroxymethyl-2′-deoxyguanosine (Figure 2).[76–81] These initial adducts lead to DNA-DNA crosslinks such as dAdo-CH₂-dAdo, dGuo-CH₂-dGuo and dAdo-CH₂-dAdo.[76, 77, 81] They also lead to the formation of DNA-protein crosslinks.[82] Formaldehyde is mutagenic in CHO, V79 and mouse lymphoma cells as well as human cells.[83–87] Formaldehyde induced AT to TA, AT to CG and GC to TA mutations in CHO cells.[85] Recent studies in CHO cells provide evidence that that formaldehyde could contribute to the mutagenic properties of nitrosamines.[88] A number of these formaldehyde DNA adducts have been detected in NNK-treated rodents and in NNKOAc-treated calf thymus DNA.[81, 89] There are no studies investigating the formation of DNA-protein crosslinks in tissue DNA following NNK exposure.

While OPB-derived DNA adducts have not been directly detected, indirect evidence for DNA damage by this aldehyde exists. It induced sister chromatid exchanges in V79 cells (OPB concentrations from 0.01 to 0.5 mM) and caused DNA single strand breaks in V79 cells (OPB concentrations from 0.01 to 1 mM) and hepatocytes (OPB concentrations > 5 mM).[90–92]

3.4. Pyridylhydroxybutyl DNA adducts

Methyl hydroxylation of NNAL leads to the formation of at least four distinct DNA adducts: 7-[4-3-(pyridyl)-4-hydroxybut-1-yl]-2′-deoxyguanosine (7-phbdGuo), O^6-[4-3-(pyridyl)-4-hydroxybut-1-yl]-2′-deoxyguanosine (O^6-phbdGuo), O^2-[4-3-(pyridyl)-4-hydroxybut-1-yl]-2′-deoxycytosine (O^2-phbdCyd), and O^2-[4-3-(pyridyl)-4-hydroxybut-1-yl]-2′-deoxythymidine (O^2-phbdThd) (Figure 2).[56, 93] The mutagenic activity of these adducts has not been investigated. However, (R)-NNAL is a pulmonary carcinogen and it generates significantly more pyridylhydroxybutyl DNA adducts than pyridyloxobutyl DNA adducts and O^6-mdGuo.[28] Therefore, the genotoxic potential of this DNA damaging pathway should be explored.

4. Contribution of specific DNA adducts to the genotoxic and carcinogenic activity of NNK in model systems

Which adduct contributes to the genotoxic effects of a nitrosamine will depend on the context. As outlined in the examples below, it depends on the relative amounts of each DNA alkylating pathway occurring in the model system, the levels and genetic variants of key repair enzymes and the gene which is targeted for mutation.

4.1. Bacterial models of genotoxicity

In most bacterial systems tested, the pyridyloxobutylating pathway was more genotoxic than the methylating pathway. Deuterium substitution on the α-methyl but not the α-methylene carbons of NNK reduced the mutagenic activity of NNK in *S. typhimurium* strains TA 1535 and TA100 in the presence of Aroclor induced rat liver S9, indicating that pyridyloxobutylation pathway as more active as a mutagen in the bacteria. [94] Consistently, model pyridyloxobutylating agents are more potent bacterial mutagens than methylating agents.[94, 95] The pyridyloxobutylating agent, 4-(carbethoxynitrosamino)-1-(3-pyridyl)-

butanone, was more mutagenic than the methylating agent carbethoxynitrosaminomethane in the same strains.[94] In the *Salmonella typhimurium* mutagenesis test, NNKOAc produced 770 – 1400 revertants/μmol in strains TA98, TA100 and TA1535 whereas the model methylating agent, acetoxymethylmethylnitrosamine (AMMN, Figure 3) caused 130 – 170 revertants/μmol in these strains.[95] These three strains are measurements of mutation frequency at GC base pairs; TA100 and TA1535 requires a point mutation whereas TA98 detects frameshift mutations.[96]

One reason that these bacterial strains are less susceptible to the mutagenic effects of methylating agents is that they have a robust repair defense against O^6-mdGuo. The *ogt* gene codes for a constitutively active form of AGT whereas the expression of the ada protein is inducible during alkylation stress. Knockout of these two proteins causes bacteria to become very sensitive to the mutagenic and toxic properties of methylating agents.[97] Studies with recombinant forms of AGT indicated that O^6-pobdGuo is a poor substrate for the equivalent bacterial repair proteins Ogt and Ada.[65] These proteins repair O^6-pobdGuo more than ten times more slowly than O^6-mdGuo, explaining, in part, the increased mutagenic activity of pyridyloxobutylating agents over methylating agents in bacterial systems. When mutagenic activity was compared to DNA adduct levels in the TA100 strain, the mutations per mutagenic adduct were comparable between NNKOAc and AMMN; all HPB-releasing adducts were assumed to be mutagenic whereas O^6-mdGuo was considered as the only mutagenic adduct formed.[95] Furthermore, the expression of human AGT in *S. typhimurium* strain YG7108 protects against the mutagenic activity of methylating and pyridyloxobutylating agents.[67, 98] This strain is a derivative of TA1535 which lacks both the *ada* and *ogt* genes detects mutations at GC base pairs and is exquisitely sensitive to mutagenic properties of methylating agents.[98] Collectively, these data support the hypothesis that the different repair rates for O^6-mdGuo and O^6-pobdGuo contributes to the different activities of methylating and pyridyloxobutylating agents in these bacterial strains.

The two O^6-alkylguanine DNA adducts are also differentially mutagenic in bacterial systems as demonstrated in site-specific mutagenesis studies in *E. coli* DH10B cells.[63, 99] O^6-pobdGuo is very mutagenic when presented as a single stranded substrate, generating a mutation rate of 96%, with all of these mutations being GC to AT mutations.[63] In contrast, O^6-mdGuo in an identical construct had a mutation rate of 60%, with all of the mutants being GC to AT mutations.[99]

Another factor likely influencing the mutation frequency of methylating and pyridyloxobutylating agents in these bacterial strains is the differing cytotoxic properties of the O^6-alkylguanine adducts. Unlike O^6-mdGuo, O^6-pobdGuo does not appear to be a cytotoxic adduct in bacteria. Modulation of adduct levels by the expression of human AGT in *S. typhimurium* strain YG7108 had no impact on the cytotoxicity of NNKOAc whereas it dramatically reduced the cytotoxic properties of a methylating agent.[67] Cytotoxicity can limit the mutagenic properties of an alkylating agent since the cells are dying and therefore, are not able to undergo mutation.

Neither NNKOAc or AMMN were very active at inducing mutations in *S. typhimurium* strains TA102, which contains an AT base pair at the reversion site.[95, 96] Recent studies

demonstrate that O^2-pobdThd blocks replication in *E. coli* which will negatively affect the mutation frequency at AT base pairs.[51] Some mutagenic activity was observed, supporting the formation of methyl and pyridyloxobutyl DNA adducts at AT base pairs. The pyridyloxobutyl DNA adduct, O^2-pobdThd, is modestly mutagenic in bacterial strains; TA to GC was the major mutation (37%) compared to TA to AT (12%) induced by this adduct.[51] The pyridyloxobutyl analog was more miscoding than the corresponding methyl analog.[51]

A more complex picture emerges when testing the mutagenesis of NNK in genes that do not require mutations at specific base pairs. For example, mutations in the *lacI* gene in *E. coli* exposed to NNK in the presence of Arochlor-induced S9 indicate that multiple mutagenic NNK-derived DNA adducts are formed. While GC to AT mutations were dominant, representing 55% of all the mutations, GC to TA (10%), GC to CG (7%), AT to GC (5%), AT to CG (5%) and AT to TA (3%), frameshifts (5%), deletions (3%) and duplication (5%).[100] Since these studies were performed in *E. coli* strains with active bacterial DNA repair and damage response genes, it is likely that O^6-pobdGuo is responsible for the GC to AT mutations and O^2-pobdThd is causing the AT to GC mutations in this gene. Similar studies with methylating agents indicate they generated roughly 90% GC to AT mutations.[101, 102]

4.2. Mammalian cell lines

In mammalian cell lines, the relative mutagenic activity of the pathways is reversed, with methylating agents often being more mutagenic than the pyridyloxobutylation pathway. In a transgenic Chinese hamster V79 cell line expressing the *Escherichia coli* xanthine-guanine phosphoribosyl transferase (*gpt*) gene (G12 cells),[103] the methylating agent AMMN was more mutagenic than the pyridyloxobutylating chemical NNKOAc.[95] Similarly, methylating agents are much more toxic on a molar basis than NNKOAc in CHO cells.[68, 88] Much of these differences is explained by differences in the levels of DNA damage;[68, 88, 95] the reactive methanediazonium ion is a more potent DNA alkylating species than an equal molar amount of a corresponding pyridyloxobutyl analog.

When looking at relative mutagenic activity of specific adducts, O^6-mG in a gapped vector is more mutagenic than O^6-pobdGuo when HEK293T cells have full repair capacity.[34, 63] The bulky O^6-pobdGuo is more mutagenic than O^6-mdGuo when AGT is depleted in these cell lines.[34, 63] The relative mutagenic properties of O^2-pobdThd and O^2-mdThd have also been compared in HEK293T cells.[52] The smaller adduct was 20% more mutagenic than the larger one in part because O^2-pobdThd was a greater block to translesional synthesis than O^2-mdThd. Both adducts primarily induce TA to AT mutations in this model.

In CHO cells, O^6-mdGuo was primarily responsible for the cytotoxic and mutagenic properties of methylating agents.[39, 40, 88] The situation is more complicated for the pyridyloxobutylation pathway. This question was explored in CHO cells with different repair capacities.[68] CHO cells do not express AGT. Expression of human AGT in these cells protected the cells against the toxic but not mutagenic effects of NNKOAc. Since the expression of AGT led to the complete removal of O^6-pobdG, it appears that this adduct does not contribute significantly to the genotoxic effects of NNKOAc in the *hprt* gene in this model. Deficiencies in NER or BER lead to both increased sensitivity of CHO cells to the

toxic and mutagenic activity of NNKOAc to a similar extent.[68] The removal of O^2-pobdThd was affected by knocking out the NER pathway; none of the other adduct levels were influenced by the absence of NER. Therefore, O^2-pobdThd likely contributes to the cytotoxicity and mutagenicity of NNKOAc. This observation is consistent with the report that this adduct blocks translesional synthesis and gives rise to TA to AT mutations with small amounts of TA to GC and/or TA to CG mutations.[52] Knockout of BER in these cells did not influence the repair of any of the pyridyloxobutyl DNA adducts since the missing protein is XRCC1 which is downstream from adduct removal in the BER pathway.

The activity of hypoxanthine-guanine phosphoribosyltransferase (hprt) can be knocked out by a wide variety of *hprt* gene mutations.[104] Sequencing of the *hprt* mutants indicated that the majority of NNKOAc-induced mutations in the hprt gene were at AT base pairs (Table 2). The expression of AGT reduced the extent of GC to AT mutations consistent with the complete repair of O^6-pobdGuo in this cell line (CHO^{AGT} cells, Table 2). This reduction did not impact the overall mutagenesis rate in this gene since GC to AT mutations were a small percentage of the overall total. Furthermore, it is possible that 7-pobdGuo and O^2-pobdCyd could contribute to the mutation frequency at GC basepairs. NNKOAc-induced TA to AT and TA to CG were significantly increased in the absence of NER (UV5 cells, Table 2). Since the repair of O^2-pobdThd was reduced in these cells, it is hypothesized to be the source of these mutations. Finally, BER protected against NNKOAc-induced hprt mutations at AT base pairs as evidenced by the increase in mutations at AT base pairs when XRCC1 is knocked out (EM9 cells, Table 2). It is not known whether O^2-pobdThd is a substrate for BER glycosylases. There may also be other uncharacterized adenine or thymidine DNA adducts. Another possibility is that formaldehyde which is generated from the decomposition of NNKOAc (Figure 3) is responsible for some of the NNKOAc-derived mutagenic DNA damage.[81] Formaldehyde induced AT to TA (3/6), AT to CG (2/6) and GC to TA (1/6) mutations in CHO cells[85] so it could be an important mutagenic adduct formed along with the pyridyloxobutyl DNA damage derived from NNKOAc.

4.3. Animal models

Investigations in animal models support the hypothesis that the DNA adducts that contribute to the carcinogenic properties of NNK depends on the model which is being tested. The relative contribution of each α-hydroxylation pathway and its associated DNA damage to the overall carcinogenic properties of nitrosamines is species and tissue dependent.

4.3.1. Mouse models—DNA methylation is the critical pathway for NNK-induced lung tumorigenesis in the A/J mouse lung tumor model.[27, 105] Compounds that only pyridyloxobutylate DNA are weak lung carcinogens in this model.[27] Consistently, deuterium substitution of the methylene protons of NNK significantly reduced the levels of O^6-mdGuo and, consequently, the pulmonary carcinogenic activity of NNK.[105] Deuteration of the methyl group caused a significant increase in lung tumorigenic activity over the unlabeled compound. A comparison of methyl and pyridyloxobutyl DNA adduct levels indicates that there is substantially more O^6-mdGuo adducts than pyridyloxobutyl DNA adducts formed in A/J mouse lungs (Figure 4), supporting the major role of the methylation pathway in the overall lung tumorigenic activity of NNK.

The formation and persistence of O^6-mdGuo is critical for NNK-induced lung tumor formation in A/J mice.[27, 106] The mutagenic adduct O^6-mdGuo persists in lung DNA for at least two weeks; these levels are highly correlated to pulmonary tumorigenic activity in A/J mice [27, 106] The persistence of this adduct is, in part, driven by the relatively poor repair capacity of the lung. The levels of AGT are much lower in the lung than the liver (Table 3).[106] Furthermore, the activity of this important repair protein is suppressed for much longer in the target tissue (Table 3).[106] The overexpression of human AGT in transgenic mice reduced the potency of NNK as a lung carcinogen.[107]

Additional support for the role of O^6-mdGuo in the tumorigenic activity of NNK in the A/J mouse comes from investigations into the K-*ras* mutation spectra induced by NNK and model alkylating compounds (Table 4). K-*ras* mutation and activation is closely associated with pulmonary tumor formation in NNK-treated A/J mice.[108, 109] Ninety six percent of the K-*ras* mutations in tumors from NNK-treated mice were GC to AT mutations in the second base of the twelfth codon, consistent with the formation of O^6-mdGuo. All of the K-*ras* mutations in tumors from AMMN-treated mice were GC to AT mutations at this loci. NNKOAc induced GC to TA as well as GC to AT mutations in the twelfth codon of the K-*ras* oncogene of tumors in A/J mice.[110] Together, these data indicate that metabolic activation of NNK to a DNA methylating agent is critical for lung tumor formation in the A/J mouse.

Point mutations in codon 12, 13 or 61 of the ras gene leads to amino acid substitution in the protein that impairs the GTPase activity of the protein, resulting in constitutive activation of ras signaling pathways, leading to tumor formation.[reviewed in 111] In the codon 12, these activating mutations primarily occur at GC base pairs.[112] Therefore, it is not surprising that alkylating agents are particularly good at generated activated K-ras mutations.

As with the bacterial mutagenesis experiments, investigations into the mutagenic properties of NNK in mice using genes that are knocked out by a broad spectrum of mutations indicate that NNK is able to trigger a more complex spectrum of mutations. Studies in Muta™ transgenic mice demonstrated that NNK was a potent inducer of *lacZ* and *cII* mutations in both lungs and liver.[113] Unlike the mutation spectrum in K-*ras* when the mutations were almost exclusively GC to AT mutations, the dominant mutation in the *cII* gene was AT to TA mutations, followed by AT to CG mutations. There was a more modest increase in GC to AT mutations at this gene loci. When the AGT activity was knocked out in this mouse strain, a modest increase in GC to AT mutations were observed in the *lacI* gene in the lungs of NNK-treated mice.[114] These data indicate that NNK is capable of generating mutations at both GC and AT base pairs in mouse lungs, indicating that multiple mutagenic adducts are formed from NNK.

While the formation of O^6-mdGuo is critical for lung tumor formation in the A/J mouse, there is strong evidence that the pyridyloxobutylation pathway also contributes to the overall tumorigenic activity of NNK in this model. When the methylating agent, AMMN, was co-administered with the pyridyloxobutylating agent, NNKOAc, the tumor yield was higher.[27] Because this combination resulted in higher levels of O^6-mdGuo, the ability of NNKOAc to increase the tumorigenic activity of AMMN was attributed to its ability to enhance the

persistence of O^6-mdGuo in lung DNA.[27] Subsequent studies indicate that this increased persistence is in part due to competition between O^6-pobdGuo and O^6-mdGuo for repair by AGT.[27, 66, 106]

Both O^6-pobdGuo and O^6-mdGuo are substrates for AGT as discussed above. Studies with purified proteins indicate that these adducts compete efficiently with one another for repair by AGT variants. [65] Therefore, the presence of both O^6-alkylguanine adducts will lead to their increased persistence in lung DNA. However, the *in vivo* levels of O^6-pobdGuo are not sufficient to explain the persistence of O^6-mdGuo in NNK-treated mouse lung. At 8 h after treatment, the levels of O^6-pobdGuo are 1/20th of those of O^6-mdGuo (Figure 4).[27, 73] Even taking into account that some repair of O^6-pobdGuo has occurred, the levels of O^6-pobdGuo are not high enough for competition for AGT to explain the extended persistence of O^6-mdGuo. Similarly, the adduct level measured for a 4.2 μmol dose of NNKOAc (3 pmol/μmol guanine) is not sufficient to explain the doubling of O^6-mdGuo levels observed in lung DNA (from 20 pmol O^6-mdGuo/μmol guanine to 41 pmol O^6-mdGuo/μmol guanine at 4 h) when a 1.0 μmol dose of AMMN is combined with 4.2 μmol dose of NNKOAc.[27, 73] Therefore, other mechanisms of interaction need to be considered. One possibility is that the pyridyloxobutylation pathway reduces the expression of AGT through an indirect route. Support for this hypothesis comes from the observation that levels of NER proteins are reduced in lungs of A/J mice following NNK exposure.[115]

Another way that the pyridyloxobutylation pathway can contribute to the tumorigenic properties of NNK is through increasing the mutagenic burden in lung DNA. Adduct studies indicate that the pyridyloxobutyl DNA persist for more than 96 h following treatment with NNK.[73] The levels of 7-pobdGuo and O^2-pobdThd did not change significantly over this period of time. Furthermore, pyridylhydroxylbutyl DNA adducts have been detected in lung DNA from NNK-treated mice.[116] These adducts result from the in vivo reduction of NNK to NNAL followed by α-methyl hydroxylation generating a pyridylhydroxybutylating agent in the lungs. The levels of O^6-hpbdGuo, O^2-hpbdThd and 7-hpbdGuo were slightly higher than the levels of pyridyloxobutyl DNA adducts but still much lower than the levels of O^6-mdGuo in the target tissue. All these bulky adducts could contribute to the tumorigenic activity of NNK by causing mutations in genes other than K-*ras*.

4.3.2. Rat models—In rats, the relative contribution of the DNA adducts to the pulmonary carcinogenic effects of NNK is more complicated. The available experimental evidence is consistent with the hypothesis that both DNA methylation and DNA pyridyloxobutylation are important for tumor formation. Compounds that generate only DNA methyl adducts (i.e dimethylnitrosamine) or only pyridyloxobutyl DNA adducts (i.e. N-nitrosonornicotine) are not potent lung carcinogens in this rodent model.[12, 24] Furthermore, [4,4-^{2}H$_2$]NNK and [C^{2}H$_3$]NNK were comparable in potency as rat pulmonary carcinogens.[117] DNA adduct studies indicate that both methyl and pyridyloxobutyl DNA adducts persist in rat lung DNA following NNK treatment. The mutagenic methyl adduct, O^6-mdGuo, was selectively persistent in Clara cells for up to 8 days after NNK treatment (10 mg/kg/day for 4 days); it is efficiently removed from DNA in other lung cell types.[118] A strong correlation between tumor formation and levels of O^6-mdGuo in Clara cells was observed in NNK-treated rats.[118] While there was not a similar correlation between O^6-mdGuo in type II cells and

tumor formation,[118] HPB-releasing DNA adduct in Type II cells were strongly correlated to lung tumor formation in rats.[119] In this study, no correlation was not observed between HPB-releasing adduct levels in Clara cells and tumor formation. Type II cells are the source of NNK-derived lung tumors in rats.[22] These data suggest that there may be communication from initiated Clara cells that influence the transformation of NNK-induced tumorigenesis in Type II cells.[25]

More recently, specific pyridyloxobutyl or pyridylhydroxybutyl DNA damage have been quantified in lung DNA of NNK-treated rats.[28, 29, 61, 89, 120] The most abundant pyridyloxobutyl adducts in lung DNA are 7-pobdGuo and O^2-pobdThd. The latter adduct accumulates more readily in chronic dosing studies.[28, 120] The levels of O^6-pobdGuo are very low, indicating that it is rapidly repaired in this animal model.[28, 29, 61, 89, 120] The levels of hydroxybutyl DNA adducts are lower than the levels of the pyridyloxobutyl DNA adducts.[28, 121] As with O^2-pobdThd, O^2-phbdT accumulates in lung DNA with chronic treatment.[121]

Formaldehyde DNA adducts as measured by the conversion of N^6-HOCH$_2$-dA to N^6-mdA were detected in lung DNA from rats treated with 4 daily s.c. injections of NNK.[89] The levels of this adduct were comparable to the levels of O^6-pobdG in these animals. Given that the extent of conversion of the adduct to the stable form analyzed for, the amounts measured are likely an underestimation of the levels of this adduct present in vivo.

A comparison of the levels of methyl, pyridyloxobutyl and pyridylhydroxylbutyl DNA adducts in lungs of rats chronically treated with 10 ppm NNK indicates that the levels of O^6-mdGuo are comparable to levels of 7-pobdGuo and O^2-pobdThd at early time points but O^2-pobdThd is more likely to accumulate in lung DNA (Figure 5).[121] A more recent study in animals receiving 5 ppm NNK indicated that O^2-pobdThd was the major adduct in lung DNA with 7-pobdGuo and O^6-mdGuo being the next most abundant adducts.[28] As in the previous study, the levels of O^6-pobdG were very low. Collectively, these experimental data support a hypothesis that O^2-pobdThd and O^6-mdGuo are the primary adducts that may contribute to the formation of lung tumors in NNK-treated rats.

5. Summary and Future Directions

Together, these data provide strong evidence for why NNK is such a potent carcinogen. There are multiple pathways that lead to the formation of a variety of genotoxic DNA adducts. Each pathway by itself has carcinogenic potential, capable of generating significant damage at both GC and AT base pairs. Which adduct will contribute to the overall carcinogenic activity depends not only on the amount formed, but the activity of the various repair pathways present at the site of DNA damage and on what types of mutations that are required to activate oncogenes or turn off tumor suppressor genes.

Smokers are exposed to and metabolize NNK.[122–124] α-Hydroxylation is the major metabolic pathway in humans[125] Evidence for both methylation and pyridyloxobutylation of DNA in smokers is available.[126–130] It is likely that these adducts contribute to the overall tumorigenic properties of tobacco smoke in humans. Which NNK adducts contribute to the

carcinogenic properties of NNK in humans is not known. Unlike rodents, human AGT repairs O^6-pobdGuo more slowly than O^6-mdGuo indicating that this mutagenic pyridyloxobutyl DNA adduct may be more persistent in humans than it is in the rodent models.[65] A detailed investigation into this question in human cell lines would provide valuable insights into this question.

Genetic variations in metabolism and DNA repair and response proteins may influence which adduct or spectrum of adducts is harmful to any one individual. Very little is known how humans vary in their sensitivity to the DNA damage generated from NNK. Given the wide variety of DNA damage coming from a single molecule, one can envision that different individuals will be more or less sensitive to specific types of NNK-derived DNA damage. For example, some epidemiological studies indicate that smoking related O^6-alkylguanine adducts contribute to lung cancer risk from tobacco smoke exposure. Genetic variations in the *mgmt* gene which codes for AGT are associated with increased risk of lung cancer in smokers or those exposed to second hand smoke.[131–133] My research group has shown that the genetic variants of AGT that impact the binding pocket size have differing abilities to repair O^6-pobdGuo versus O^6-mdGuo *in vitro*.[65, 134] Understanding how these in vitro differences translate into susceptibility to mutagenesis requires further study.

A complete understanding of the role of specific adducts to the carcinogenic activity of NNK will also require a more complete understanding of the toxicological properties of specific DNA damage. The mutagenic characteristics of 7-pobdGuo should be defined. This adduct persists in vivo and may contribute to NNK-derived mutations at GC base pairs. Furthermore, it should be determined if this adduct can undergo imidazole ring opening to mutagenic fapy adducts; methyl-fapy and aflatoxin B1-fapy adducts caused GC to TA transversion mutations, providing support that a pyridyloxobutyl fapy adduct might be an important NNK-derived DNA damage.[70–72] The role of aldehyde DNA damage in NNK-induced mutagenesis and carcinogenesis should also be defined. Our studies in CHO cells indicate that these adducts may play an important role in the toxicological properties of NNK. Finally, the genotoxic properties of the pyridyloxobutyl phosphate adducts as well as all of the pyridylhydroxybutyl DNA adducts are still to be explored.

Acknowledgments

Abbreviations

7-mdGuo	7-methyl-2′-deoxyguanosine
7-phbdGuo	7 [4-3-(pyridyl)-4-hydroxybut-1-yl]-2′-deoxyguanosine
7-pobGua	7-[4-3-(pyridyl)-4-oxobut-1-yl]guanine

7-pobdGuo	7-[4-3-(pyridyl)-4-oxobut-1-yl]-2′-deoxyguanosine
AGT	O^6-alkylguanine DNA alkyltransferase
AMMN	*N*-acetoxymethyl-*N*-methylnitrosamine
BER	base excision repair
CHO	Chinese hamster ovary
fapy	formamidopyrimidine
HEK293T cells	human embryonic kidney 293T cells
HPB	4-hydroxy-1-(3-pyridyl)-1-butanone
hprt	hypoxanthine-guanine phosphoribosyl transferase
MMR	mismatch repair
NER	nucleotide excision repair
NNAL	4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol
NNK	4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone
NNKOAc	4-(acetoxymethylnitrosamino)-1-(3-pyridyl)-1-butanone
***O²*-phbdCyd**	O^2-[4-3-(pyridyl)-4-hydroxybut-1-yl]-2′-deoxycytosine
***O²*-pobdCyd**	O^2-[4-3-(pyridyl)-4-oxobut-1-yl]-2′-deoxycytosine
***O²*-pobCyt**	O^2-[4-3-(pyridyl)-4-oxobut-1-yl]cytosine
***O⁶*-mdGuo**	O^6-methyl-2′-deoxyguanosine
***O⁶*-phbdGuo**	O6-[4-3-(pyridyl)-4-hydroxybut-1-yl]-2′-deoxyguanosine
***O⁶*-pobGua**	O^6-[4-3-(pyridyl)-4-oxobut-1-yl]-guanine
***O⁶*-pobdGuo**	O6-[4-3-(pyridyl)-4-oxobut-1-yl]-2′-deoxyguanosine
***O²*-mdThd**	O^2-methyl-2′-deoxythymidine
***O⁴*-mdThd**	O^4-methyl-2′-deoxythymidine
O²-phbdThd	O^2-[4-3-(pyridyl)-4-hydroxybut-1-yl]-2′-deoxythymidine
***O²*-pobdThd**	O2-[4-3-(pyridyl)-4-oxobut-1-yl]-2′-deoxythymidine
***O²*-pobdThy**	O^2-[4-3-(pyridyl)-4-oxobut-1-yl]-thymine
OPB	4-oxo-1-(3-pyridyl)-1-butanone

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Biography

Lisa Petersons is a professor in the Division of Environmental Health Sciences and co-Leader of the Carcinogenesis and Chemoprevention program in the Masonic Cancer Center, University of Minnesota. Her PhD is in Pharmaceutical Chemistry from University of California, San Francisco where she studied with Neal Castagnoli, Jr. After postdoctoral studies with Fred Guengerich at Vanderbilt University, she joined Stephen Hecht's group at the American Health Foundation in Valhalla, NY. In 1997, Lisa moved to the University of Minnesota. Her research focuses on mechanisms by which chemicals initiate carcinogenesis. She is an Associate Editor for *Chemical Research in Toxicology*
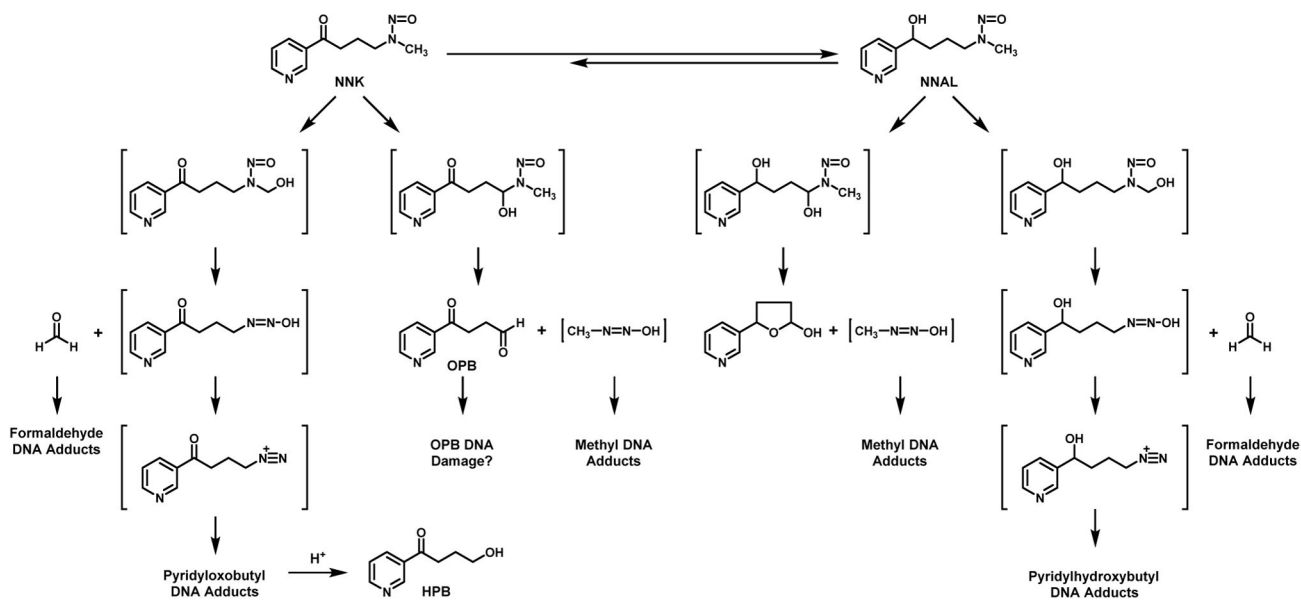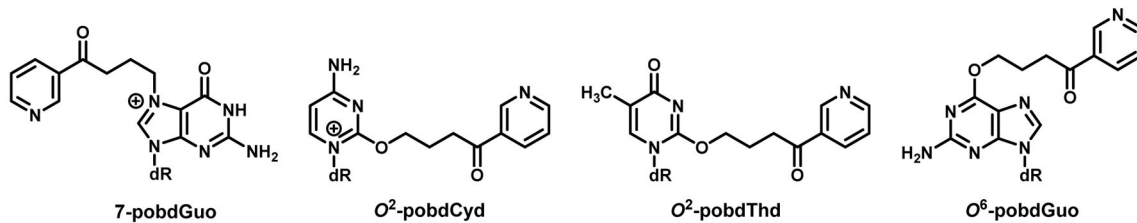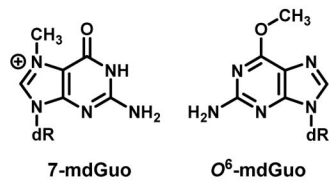
Figure 1.
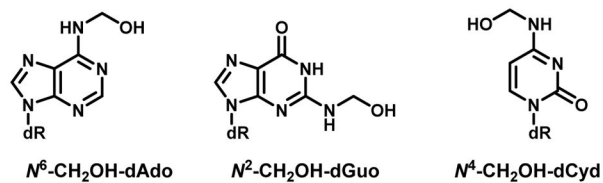Proposed bioactivation pathways for NNK and its metabolite, NNAL.

Pyridyloxobutyl DNA adducts

7-pobdGuo O^2-pobdCyd O^2-pobdThd O^6-pobdGuo

Methyl DNA adducts   **Formaldehyde DNA adducts**

7-mdGuo O^6-mdGuo N^6-CH$_2$OH-dAdo N^2-CH$_2$OH-dGuo N^4-CH$_2$OH-dCyd

Pyridylhydroxybutyl DNA adducts

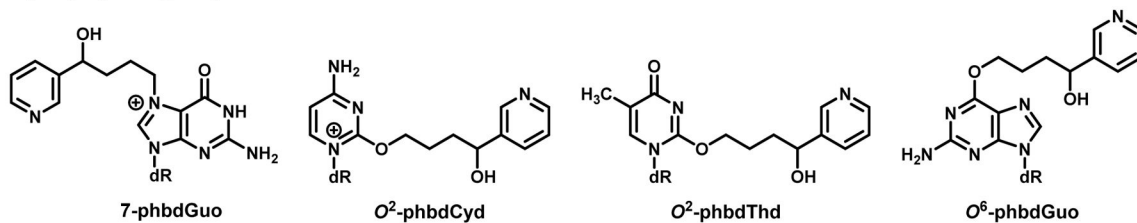7-phbdGuo O^2-phbdCyd O^2-phbdThd O^6-phbdGuo

Figure 2.
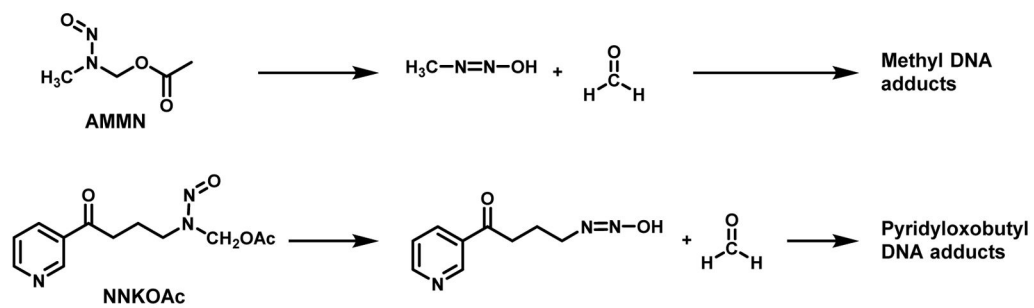Structures of NNK-derived DNA adducts.

Figure 3.
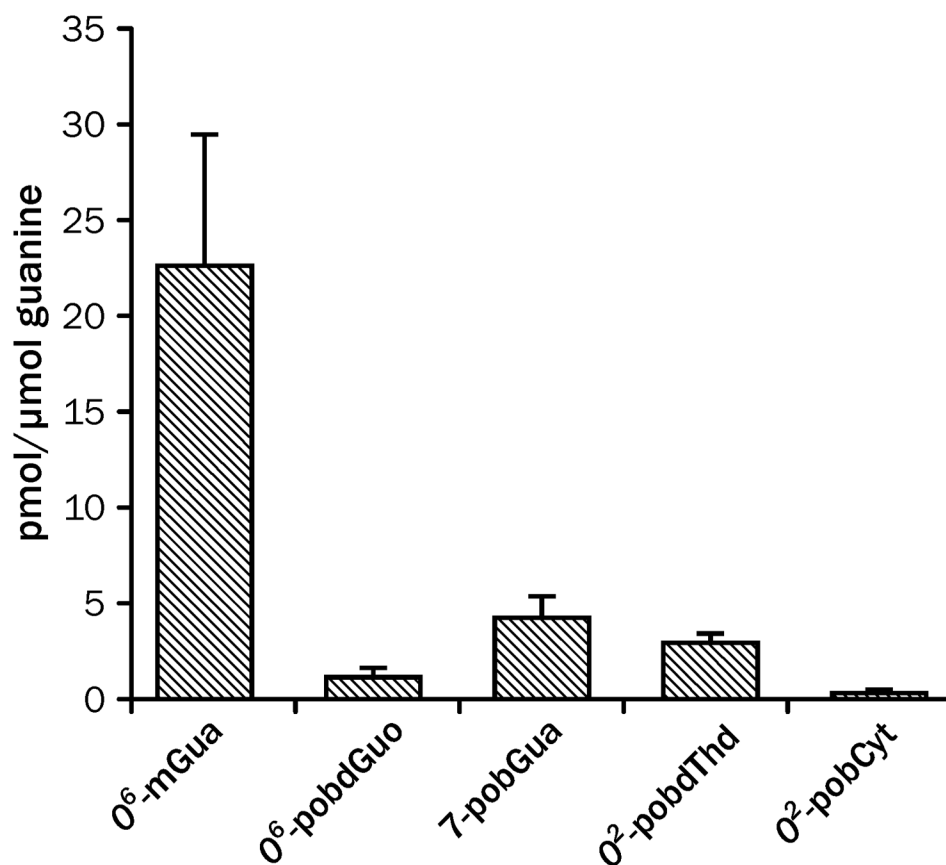Structures and hydrolysis of model alkylating agents.

Figure 4.
Levels of NNK-derived DNA damage in lungs of A/J mice 8 h after treatment with 10 μmol NNK.[27, 73] The error bars represent standard deviation of 3 – 5 replicates.
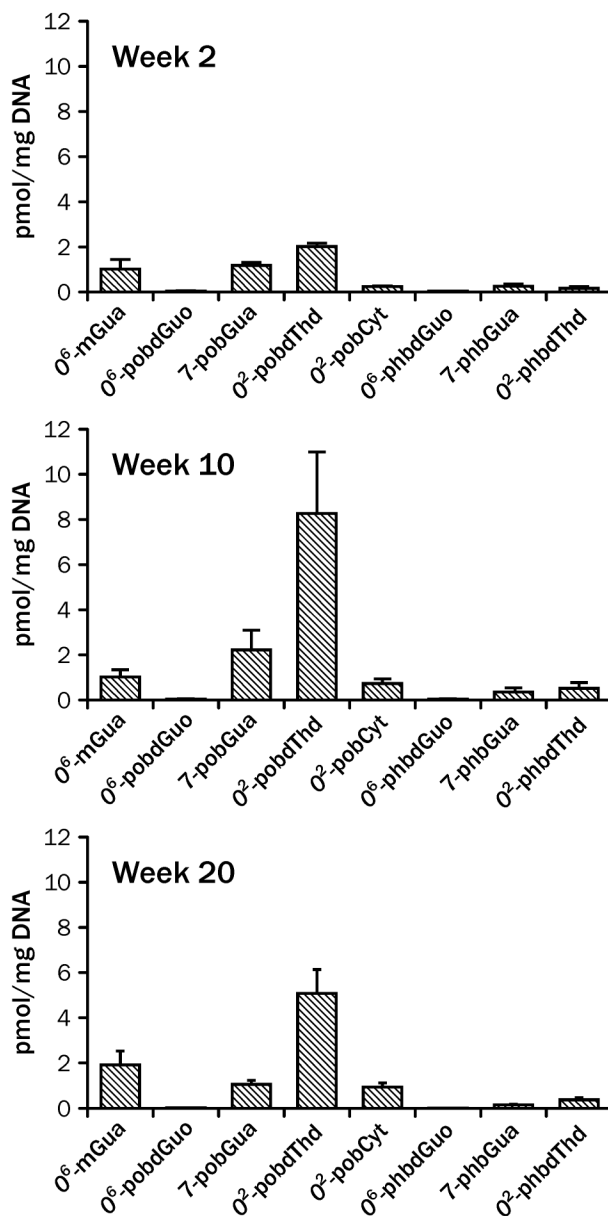
Figure 5.
Levels of adducts in lungs of rats treated with 10 ppm NNK in the drinking water for 2, 10 and 20 weeks.[29, 120, 121] The error bars represent standard deviation of 3 replicates

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Table 1

Stability of pyridyloxobutyl DNA adducts

DNA adduct	Conditions	product	reference
O^6-pobdGuo	pH 7, 30 min at 100° C	Stable	54
	0.1 N HCl, 30 min at 80° C	O^6-pobGua	54
	0.8 N HCl, 1 h at 80° C	HPB and Gua	54
7-pobdGuo	Enzyme hydrolysis of DNA: pH 7, 70 min at 37° C	Stable	55
	pH 7, 1 h at 100° C	7-pobGua and HPB	55
	0.1 N HCl, 1 h at 80° C	7-pobGua and HPB	55
O^2-pobdCyd	Enzyme hydrolysis of DNA: pH 7, 70 min at 37° C	Stable	56
	pH 7, 1 h at 100° C	O^2-pobCyt and HPB	56
O^2-pobdThd	Enzyme hydrolysis of DNA: pH 7, 70 min at 37° C	Stable	56
	pH 7, 1 h at 100° C	Stable	56
	0.1 N HCl, 1 h at 85° C	O^2-pobThy	56
Phosphate adducts	pH 7, 48 h at 37° C	Stable	58

Table 2

Base substitution mutations observed in 6-thioguanine-resistant mutant colonies in CHO cell lines.[68]

	% of base substitution						
	24 µM NNKOAc					Spontaneous	
Class of mutations	AA8[1]	UV5	EM9	CHOpcDNA3	CHOAGT	AA8[2]	EM9[3]
Transitions:							
A:T→G:C	4	13	13	13	12	2	0
G:C→A:T	25	17	17	17	5	16	16
Transversions:							
A:T→C:G	63	35	52	70	74	12	0
A:T→T:A	4	22	17	0	5	6	8
G:C→C:G	0	4	0	0	0	12	8
G:C→T:A	4	9	0	0	5	35	69

[1] The parental cell line, AA8, does not express the repair protein AGT [135]. UV5 has a mutation in the ERCC-2 gene (XPD) and, as a result, is deficient in NER. [136]. EM9 lacks XRCC1 and is compromised in BER. [137] CHOpcDNA3 cells are derived from AA8 cells and are stably transfected with the empty pCMV-neo-*Bam* vector. [135] CHOAGT cells are stably transfected with the pCMV-hAGT expressing human AGT. [135]

[2] As previously reported.[138]

[3] As previously reported.[139]

Table 3

Levels of AGT in lung and liver in A/J mice treated with NNK. [106]

Tissue	Treatment[a]	fmol AGT/mg protein[b]	
		4 h	**96 h**
Lung	0 μmol NNK	28 ± 5	33 ± 6
	10 μmol NNK	7 ± 2	8 ± 2
Liver	0 μmol NNK	110 ± 30	110 ± 20
	10 μmol NNK	0 ± 0	110 ± 30

[a]Groups of 30 mice were given i.p. injections NNK (0 or 10 umol) in saline (0.2 mL). Each group was subdivided into 2 groups for sacrifice at 4 or 96 h.

[b]Mean of 4 - 13 samples ± SD.

Table 4

K-ras mutations in tumors from carcinogen-treated A/J mice.[110]

Compound	No. of tumors	GGT (wt)	GAT	TGT	GTT
NNK	28	0 %	96%	4%	0%
NNKOAc	21	19%	38%	24%	19%
AMMN	18	0	100%	0	0