

## Review Article

# Formation, Repair, and Genotoxic Properties of Bulky DNA Adducts Formed from Tobacco-Specific Nitrosamines

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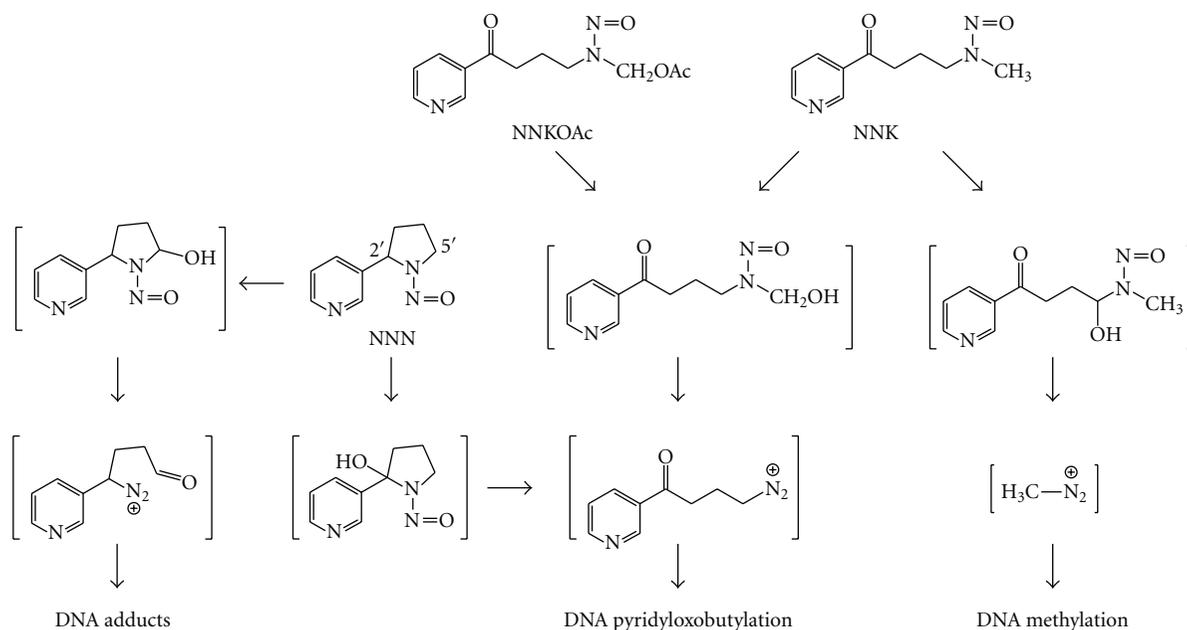
4-(Methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) and *N'*-nitrosornicotine (NNN) are tobacco-specific nitrosamines present in tobacco products and smoke. Both compounds are carcinogenic in laboratory animals, generating tumors at sites comparable to those observed in smokers. These Group 1 human carcinogens are metabolized to reactive intermediates that alkylate DNA. This paper focuses on the DNA pyridyloxobutylation pathway which is common to both compounds. This DNA route generates 7-[4-(3-pyridyl)-4-oxobut-1-yl]-2'-deoxyguanosine, *O*<sup>2</sup>-[4-(3-pyridyl)-4-oxobut-1-yl]-2'-deoxycytosine, *O*<sup>2</sup>-[4-(3-pyridyl)-4-oxobut-1-yl]-2'-deoxythymidine, and *O*<sup>6</sup>-[4-(3-pyridyl)-4-oxobut-1-yl]-2'-deoxyguanosine as well as unstable adducts which dealkylate to release 4-hydroxy-1-{3-pyridyl}-1-butanone or depyriminidate/depurinate to generate abasic sites. There are multiple repair pathways responsible for protecting against the genotoxic effects of these adducts, including adduct reversal as well as base and nucleotide excision repair pathways. Data indicate that several DNA adducts contribute to the overall mutagenic properties of pyridyloxobutylating agents. Which adducts contribute to the carcinogenic properties of this pathway are likely to depend on the biochemistry of the target tissue.

## 1. Introduction

Tobacco use has been linked to a variety of human cancers, including lung, oral cavity, esophagus, pharynx, larynx, urinary bladder, pancreas, and liver cancers [1]. Lung cancer alone is responsible for the deaths of 1.3 million people annually worldwide [2]. It is the leading cause of cancer deaths in the United States, with 80%–90% of this cancer associated with tobacco use [1]. Environmental tobacco smoke (second-hand smoke) has also been associated with human lung cancer but the risks are significantly lower than those associated with smoking [1].

There are more than 5000 identified chemicals present in cigarette smoke [1, 3–5]. More than 60 of these compounds are demonstrated chemical carcinogens in animal models [1, 3, 4, 6]. An important group of tobacco carcinogens are the tobacco-specific nitrosamines. These compounds are formed from tobacco alkaloids like nicotine during the curing process of tobacco [7]. 4-(Methylnitrosamino)-1-(3pyridyl)-1-butanone (NNK) and *N'*-nitrosornicotine (NNN) are

two of the most potent tobacco-specific nitrosamines present in tobacco products and smoke [8]. Both compounds are carcinogenic in laboratory animals, generating tumors at sites comparable to those observed in smokers [8]. NNK is a potent lung carcinogen, which also induces liver and nasal tumors [9–11]. This compound induces lung adenocarcinomas in rodents at lifetime doses that are comparable to those experienced by smokers [8]. Adenocarcinoma is now the most common type of lung cancer observed in humans, having surpassed squamous cell carcinoma [12–16]. This shift in histology has been attributed not to improvements in diagnoses but rather to changing cigarette design, which has changed smoking behavior resulting in increased uptake of tobacco-specific nitrosamines by smokers [14]. Metabolic products of NNK have been detected in urine of smokers and individuals exposed to second-hand smoke, indicating that humans are exposed to and metabolize this carcinogen [17–20]. NNN is carcinogenic to the esophagus, nasal cavity, and respiratory tract in laboratory animals [8]. This nitrosamine is present in higher amounts than any other esophageal



SCHEME 1: Pathways of bioactivation of NNK, NNN, and model pyridyloxobutylating agent, NNKOAc.

carcinogen in tobacco smoke [8]. It and/or its glucuronide conjugate have been detected in the urine and toenails of smokers and smokeless tobacco users [21–25]. Based on animal studies, NNK and NNN are listed as Group 1 human carcinogens by the International Agency for Cancer Research [6, 8].

NNK and NNN require metabolism to exert their toxicological properties [8]. NNK-induced carcinogenesis requires cytochrome P450 catalyzed metabolic activation to DNA reactive metabolites [26]. NNK is metabolized to either a methylating or a pyridyloxobutylating agent (Scheme 1). The methylation pathway generates well-characterized methyl DNA adducts, such as 7-methylguanine (7-mG),  $O^6$ -methylguanine ( $O^6$ -mG), and  $O^4$ -methylthymidine ( $O^4$ -mT) [27–31]. The dominant mutagenic adduct is  $O^6$ -mG [32, 33]. The repair mechanisms and genotoxic properties of this adduct have been extensively reviewed [34–37] and will not be a focus of this paper. The formation, repair, and genotoxic properties of the pyridyloxobutyl adducts will be discussed below.

NNN also has two pathways to form DNA adducts, 2'- and 5'-hydroxylation [8]. (*S*)-NNN, the dominant enantiomer in tobacco products [38], undergoes primarily 2'-hydroxylation whereas (*R*)-NNN undergoes both 2'- and 5'-hydroxylation [39]. 2'-Hydroxylation generates the same pyridyloxobutylating agent as methyl hydroxylation of NNK (Scheme 1). 5'-Hydroxylation generates a reactive metabolite that can also alkylate DNA (Scheme 1) [40, 41]. However, no data exist for the levels of these adducts *in vivo*. For the purpose of this paper, we will focus on the pyridyloxobutylation pathway.

## 2. Structure of Pyridyloxobutyl DNA Adducts

The pyridyloxobutylation pathway leads to a variety of adducts, four of which have been recently identified (Scheme 2). They are 7-[4-(3-pyridyl)-4-oxobut-1-yl]-2'-deoxyguanosine (7-pobdG) [42],  $O^2$ -[4-(3-pyridyl)-4-oxobut-1-yl]-2'-deoxycytosine ( $O^2$ -pobdC) [43],  $O^2$ -[4-(3-pyridyl)-4-oxobut-1-yl]-2'-deoxythymidine ( $O^2$ -pobdT) [43], and  $O^6$ -[4-(3-pyridyl)-4-oxobut-1-yl]-2'-deoxyguanosine ( $O^6$ -pobdG) [42–44]. Both 7-pobdG and  $O^2$ -pobdC readily release the corresponding nucleobases, 7[4-(3-pyridyl)-4-oxobut-1-yl]guanine (7-pobG) and  $O^2$ -[4-(3-pyridyl)-4-oxobut-1-yl]cytosine ( $O^2$ -pobC), respectively, leaving behind an abasic site [42, 43]. In addition, some pyridyloxobutyl DNA adducts are unstable and dealkylate to release 4-hydroxy-1-(3-pyridyl)-1-butanone (HPB) (Scheme 2) [31, 45]. HPB-releasing adducts include  $O^2$ -pobdC [43] and 7-pobdG [42]. Quantitation of the specific pyridyloxobutyl DNA adducts in calf thymus DNA treated with a model pyridyloxobutylating agent, 4-(acetoxymethylnitrosamino)-1-(3-pyridyl)-1-butanone (NNKOAc, Scheme 1), demonstrates that HPB-releasing adducts are the major adducts present in pyridyloxobutylated DNA [46]. They represent approximately 65% of the total adducts formed. The relative levels of the specific adducts making up the remainder are 7-pobG >  $O^6$ -pobdG >  $O^2$ -pobdT  $\geq$   $O^2$ -pobC.

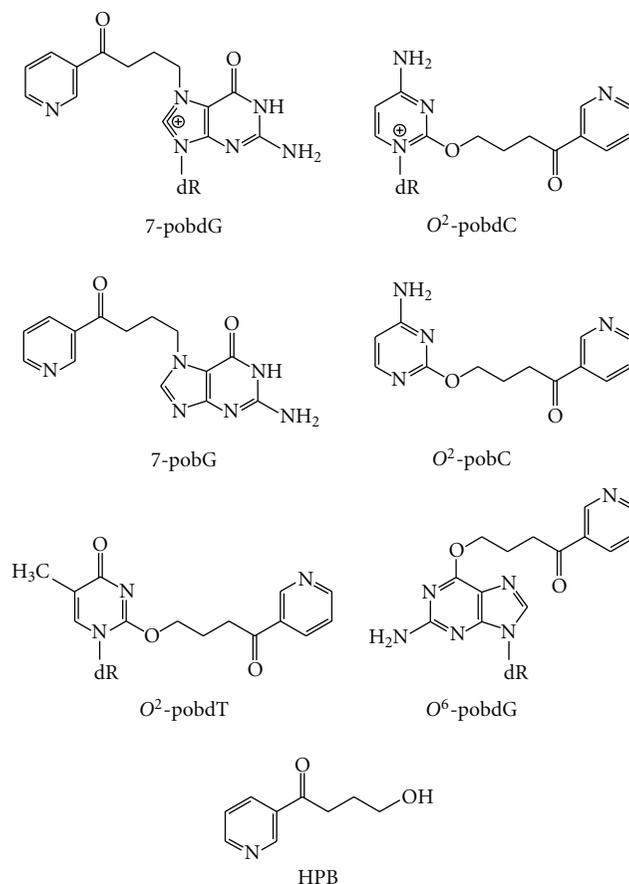
Conflicting evidence exists for the formation of phosphate adducts in pyridyloxobutylated DNA. HPB is not released from pyridyloxobutylated DNA when heated under basic conditions [45]. This observation is not consistent with the presence of pyridyloxobutyl phosphate esters. However, the 3'-termini of NNKOAc-induced strand breaks

are resistant to  $^{32}\text{P}$ -endlabeling in the presence of T4 DNA polymerase even after incubating with endonuclease IV which removes 3'-phosphate or 3'-phosphoglycolate groups [47]. This observation suggests that there may be an adduct on the 3'-phosphate group. However, the nucleobase adduct,  $O^6$ -pobdG, has been reported to inhibit 3'-exonuclease degradation of DNA [48]. Therefore, it is possible this adduct or other pyridyloxobutyl DNA adducts inhibits endonuclease IV as well. Also supporting the formation of pyridyloxobutyl phosphate adducts is the detection of a 4-(3-[ $^3\text{H}$ ]pyridyl)-4-hydroxy-2-butylcobalam complex when enzymatic digests of DNA from [ $^3\text{H}$ ]NNK-treated animals were reacted with cob(I)alamin followed by sodium borohydride [49]. This reaction product accounted for up to 22% of the total pyridyloxobutyl adducts detected. Cob(I)alamin selectively reacts with alkyl phosphate adducts [50]. However, the pyridyloxobutyl group might be more reactive with this reagent than a simple alkyl group and the product may be formed from adducts other than alkyl phosphates. This possibility requires further testing.

### 3. Levels of Pyridyloxobutyl DNA Adducts in NNK- or NNN-Treated Rodents

Pyridyloxobutyl DNA adducts have been observed in DNA isolated from the tissues of NNK- or NNN-treated animals. HPB-releasing adducts have been detected in target tissues and have been shown to persist [8, 52]. They have also been linked to tumor formation in the rat [53]. More recent studies have reported the levels of specific adducts in target and nontarget tissues of NNK- or NNN-treated rodents. One of the first studies demonstrated that  $O^6$ -pobdG was present at very low levels in lung and liver DNA from [ $^3\text{H}$ ]NNK-treated A/J mice [54]. Subsequent experiments have employed sensitive LC-MS/MS assays [55, 56] for their detection of DNA from *in vivo* sources. Table 1 displays the levels of pyridyloxobutyl DNA adducts detected in lung and liver DNA following four subcutaneous doses of NNK [51]. In this study, the relative adduct distribution was  $O^2$ -pobdT  $\geq$  7-pobG  $>$   $O^2$ -pobC  $\gg$   $O^6$ -pobdG in lung DNA and  $O^2$ -pobdT = 7-pobG  $\geq$   $O^2$ -pobC  $\gg$   $O^6$ -pobdG in liver DNA. The levels of 7-pobG,  $O^2$ -pobC and  $O^2$ -pobdT were higher in liver relative to lung DNA whereas the levels of  $O^6$ -pobdG were higher in lung relative to liver.  $O^2$ -pobdT was also the dominant adduct detected when rats were chronically treated with a lower dose of NNK (10 ppm in drinking water) (Table 2) [57, 58]. The relative distribution of pyridyloxobutyl DNA adducts was  $O^2$ -pobdT  $>$  7-pobG  $\gg$   $O^2$ -pobC  $\gg$   $O^6$ -pobdG in lung DNA and  $O^2$ -pobdT  $\gg$  7-pobG  $>$   $O^2$ -pobC in liver DNA;  $O^6$ -pobdG was not observed in liver DNA from these animals [57]. Pyridyloxobutyl DNA adducts were also observed in nasal respiratory mucosa, nasal olfactory mucosa, oral mucosa, and pancreas from NNK-treated rats [59]. The relative levels of total pyridyloxobutyl DNA adducts is lung  $>$  liver  $>$  nasal respiratory mucosa  $>$  nasal olfactory mucosa  $\approx$  oral mucosa  $\approx$  pancreas [59].

Similar studies have been performed in NNN-treated rats [60, 61]. Chronic treatment of F344 rats with (*R*)-



SCHEME 2: Structures of pyridyloxobutyl DNA adducts and HPB.

NNN or (*S*)-NNN in the drinking water (10 ppm, 1–20 weeks) led to adduct formation in lungs, liver, nasal respiratory mucosa, nasal olfactory, and oral mucosa [60, 61]. Target tissues (nasal olfactory, respiratory mucosa, and esophagus) had the highest levels of pyridyloxobutyl DNA adducts whereas the nontarget tissues (lung and liver) had the lowest levels. The enantiomers gave different levels of pyridyloxobutyl DNA adducts in the various tissues. (*R*)-NNN produced the highest levels in lung nasal olfactory and nasal respiratory tissue whereas (*S*)-NNN generated higher levels in esophagus, liver, and oral mucosa [60, 61]. These tissue-dependent differences are likely due to tissue differences in the cytochrome P450 enzymes responsible for the bioactivation of these two enantiomers [60, 61].

As with NNK,  $O^2$ -pobdT was a major adduct observed in DNA from various NNN-exposed tissues such as nasal olfactory mucosa ( $O^2$ -pobdT  $>$  7-pobG  $\gg$   $O^2$ -pobC  $>$   $O^6$ -pobdG), respiratory mucosa ( $O^2$ -pobdT  $>$  7-pobG  $\gg$   $O^2$ -pobC  $>$   $O^6$ -pobdG), and oral mucosa ( $O^2$ -pobdT  $\approx$  7-pobG  $\gg$   $O^2$ -pobC  $>$   $O^6$ -pobdG) as well as liver and lung ( $O^2$ -pobdT  $\gg$  7-pobG  $\geq$   $O^2$ -pobC). In the rat esophagus, 7-pobG was the dominant adduct (7-pobG  $\geq$   $O^2$ -pobdT  $\approx$   $O^2$ -pobC).  $O^6$ -pobdG was not detected in lung, liver or esophageal DNA [60].

TABLE 1: Adduct levels in NNK-treated rats [51].

Tissue	NNK Dose (mmol/kg) <sup>a</sup>	Mean $\pm$ S.D., $N = 5$ (fmol/mg DNA)			
		7-pobG	O <sup>2</sup> -pobdT	O <sup>2</sup> -pobC	O <sup>6</sup> -pobG
Lung	saline control	N.D. <sup>b</sup>	N.D.	N.D.	N.D.
	0.025	933 $\pm$ 89	1120 $\pm$ 66	483 $\pm$ 36	251 $\pm$ 26
	0.1	1800 $\pm$ 478	2020 $\pm$ 483	840 $\pm$ 169	487 $\pm$ 101
Liver	saline control	N.D.	N.D.	N.D.	N.D.
	0.025	3550 $\pm$ 1600	3530 $\pm$ 725	2930 $\pm$ 521	28 $\pm$ 17
	0.1	12200 $\pm$ 1600	12300 $\pm$ 1690	7800 $\pm$ 1680	140 $\pm$ 25

<sup>a</sup>Administered by s.c. injection daily for 4 days.

<sup>b</sup>N.D.: not detected (detection limit, 3 fmol/mg DNA).

#### 4. Formation of Pyridyloxobutyl DNA Adducts in Humans

While there is no information regarding the levels of the four individual pyridyloxobutyl DNA adducts in humans, HPB-releasing adducts have been detected in human tissue samples. Levels of these adducts were significantly higher ( $P < .0001$ ) in self-reported smokers who had lung cancer than in self-reported nonsmokers who had lung cancer ( $404 \pm 258$  versus  $59 \pm 56$  fmol HPB released/mg DNA, resp.) [62]. Since HPB-releasing adducts accumulate in normal lung tissues of lung cancer patients but not in normal smoking controls [62, 63], these data support a hypothesis that smokers who accumulate pyridyloxobutyl DNA adducts may be at increased risk of lung cancer.

#### 5. Repair Pathways for Pyridyloxobutyl DNA Adducts

DNA adduct repair protects a cell against the toxic and genotoxic effects of DNA damage. There are multiple pathways involved in the removal of alkylated DNA bases generated by reactive alkanediazohydroxides. These include direct base repair by alkyltransferases and excision of the DNA damage by base excision repair (BER) or nucleotide excision repair (NER). Mismatch repair is involved in the detection and repair of mismatched DNA adducts. Below is a review of the pathways thought to be involved in the repair of pyridyloxobutyl DNA damage.

**5.1. Adduct Reversal.** O<sup>6</sup>-Alkylguanine DNA alkyltransferase (AGT) is a suicide protein that repairs O<sup>6</sup>-alkylguanine adducts by facilitating the transfer of the alkyl group from the O<sup>6</sup>-position of guanine to a cysteine residue in the protein's active site [35]. This alkylation reaction inactivates the protein and triggers a conformational change [64] which leads to its degradation [65]. Consequently, the initial repair capacity of a cell is determined by its constitutive levels of AGT.

While O<sup>6</sup>-pobdG is readily repaired by mammalian AGTs, it is not a good substrate for the bacterial AGTs *ada*

and *ogt* [66]. The ability of AGT orthologs to repair this bulky O<sup>6</sup>-alkylguanine adduct is likely determined by the size of the protein's adduct binding site. Rodent AGT has the largest binding site and repairs O<sup>6</sup>-pobdG faster than human AGT which has a smaller binding pocket [66]. The bacterial AGTs have an even smaller binding pocket, explaining the inability of these proteins to repair this damage [66]. This adduct reversal pathway is a major repair pathway for O<sup>6</sup>-pobdG in mammalian cells [54, 66, 67].

**5.2. Base Excision Repair.** Base excision repair (BER) is another important pathway for the repair of nitrosamine-derived DNA damage. This pathway is involved in the repair of single strand breaks, small alkyl guanine damage, and oxidized DNA bases as well as abasic sites [68, 69]. It is a multistep process that is initiated when damaged bases are removed by glycosylases, leaving abasic sites in DNA. The abasic sites are removed by an endonuclease. The missing nucleoside is then replaced and ligation occurs. It is likely that NNK-derived methyl adducts such as 7-methylguanine and N<sup>3</sup>-methyladenine are removed by base excision repair [70]. The ability of pyridyloxobutyl adducts to serve as substrates for BER glycosylases has not been studied. It is possible that they could serve as substrates since the structurally similar adduct, O<sup>6</sup>-butylguanine, appears to be repaired in part by BER *in vivo* [71]. It is likely that abasic sites formed by the depurination/depyrimidination of 7-pobG and O<sup>2</sup>-pobC, respectively, are repaired by this pathway.

While little is known about the role of BER in the repair of pyridyloxobutyl DNA damage, two observations suggest that BER may be important. First, incubation of lysate from NNKOAc-treated cells with formamidopyrimidine glycosylase prior to the COMET assay results in a small but significant increase in strand breaks [72]. This observation indicates that there are pyridyloxobutyl DNA adducts that are substrates for this glycosylase. Second, loss of XRCC1, an important scaffold protein in BER [73], increases the mutagenic and toxic effects of NNKOAc [67]. The loss of this protein does not affect the rate of removal of specific pyridyloxobutyl DNA adducts from DNA [67]. However, the observed increase in toxicity and mutagenicity indicates

TABLE 2: Comparative DNA adduct levels in lung and liver of F344 rats treated with 10 ppm NNK in the drinking water and sacrificed at various intervals [57, 58].

		Adduct Levels fmol/mg DNA (mean $\pm$ S.D.)					
Week		1	2	5	10	16	20
Lung	O <sup>6</sup> -mG	976 $\pm$ 342	1020 $\pm$ 423	2550 $\pm$ 263	1020 $\pm$ 314	729 $\pm$ 57.5	1910 $\pm$ 615
	O <sup>6</sup> -pobdG	45 $\pm$ 7 <sup>a</sup>	50 $\pm$ 5 <sup>a</sup>	46 $\pm$ 13 <sup>a</sup>	44 $\pm$ 14 <sup>a</sup>	34 $\pm$ 17 <sup>a</sup>	20 $\pm$ 5 <sup>a</sup>
	7-pobG	750 $\pm$ 95	1180 $\pm$ 131	1360 $\pm$ 214 <sup>a</sup>	2220 $\pm$ 864	1700 $\pm$ 175 <sup>a</sup>	1060 $\pm$ 169
	O <sup>2</sup> -pobdT	1080 $\pm$ 99	2020 $\pm$ 150	3890 $\pm$ 648	8260 $\pm$ 2730 <sup>a</sup>	6720 $\pm$ 606 <sup>a</sup>	5070 $\pm$ 1060 <sup>a</sup>
	O <sup>2</sup> -pobC	240 $\pm$ 23	250 $\pm$ 18	400 $\pm$ 87 <sup>a</sup>	730 $\pm$ 211	810 $\pm$ 152	940 $\pm$ 175
Liver	Week	1	2	5	10	16	20
	O <sup>6</sup> -mG	3830 $\pm$ 865	7120 $\pm$ 2080	2310 $\pm$ 946	564 $\pm$ 250	637 $\pm$ 59	891 $\pm$ 379
	O <sup>6</sup> -pobdG	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	7-pobG	490 $\pm$ 104 <sup>a</sup>	880 $\pm$ 182 <sup>a</sup>	1050 $\pm$ 90	1460 $\pm$ 625	1170 $\pm$ 86 <sup>a</sup>	730 $\pm$ 225
	O <sup>2</sup> -pobdT	650 $\pm$ 121 <sup>a</sup>	1230 $\pm$ 272 <sup>a</sup>	2190 $\pm$ 174	3740 $\pm$ 1170 <sup>a</sup>	3540 $\pm$ 643 <sup>a</sup>	2680 $\pm$ 643 <sup>a</sup>
O <sup>2</sup> -pobC	170 $\pm$ 43 <sup>a</sup>	140 $\pm$ 25 <sup>a</sup>	240 $\pm$ 17	580 $\pm$ 214	350 $\pm$ 152	490 $\pm$ 146	

n.d., not detected.

<sup>a</sup>Significantly different from O<sup>6</sup>-mG,  $P < .05$ .

that XRCC1 plays an important role in protecting a cell against the harmful effects of these adducts. Together, these observations provide evidence for the role of BER in the repair of pyridyloxobutyl DNA damage.

**5.3. Nucleotide Excision Repair.** Another important pathway for the repair of bulky DNA damage is nucleotide excision repair (NER) [74, 75]. Like BER, NER is a multiprotein mediated repair pathway. However, in this pathway a whole section of the damaged DNA strand is removed in several steps. A new strand is then synthesized by DNA polymerase using the undamaged strand as a template.

Several pieces of experimental data support the importance of NER in the repair of pyridyloxobutyl DNA adducts. In one study, [ $\alpha$ -<sup>32</sup>P]TTP was incorporated into NNKOAc-treated plasmid DNA when incubated with extracts from normal human lymphoid cells in an ATP-dependent fashion [76]. This activity was significantly lower in cell extracts from XPA- and XPC-deficient cell lines. XPA and XPC are two important proteins involved in the initiation of the NER pathway [74, 75] so their absence significantly impacts the efficiency of NER.

A second study examined the removal of specific pyridyloxobutyl DNA adducts from DNA in NNKOAc-treated Chinese hamster ovary cells [67]. The rate of removal of these adducts was compared between the parental cell line, AA8, which has functional NER but not AGT, and UV5 cells which lacks both functional NER [loss of ERCC-2 (XPD)] and AGT [77]. O<sup>2</sup>-pobdT was the only adduct whose removal was affected by the loss of ERCC-2. Its repair was significantly slower in the absence of this protein, suggesting the importance of NER in the removal of this adduct. Since there were several reports indicating that larger

O<sup>6</sup>-alkylguanine adducts appear to be preferentially repaired by nucleotide excision repair [78–82], O<sup>6</sup>-pobdG repair was also expected to be reduced in cells lacking NER. However, O<sup>6</sup>-pobdG was a poor substrate for this pathway in CHO cells as well as in an *in vitro* human NER repair assay [67].

**5.4. Mismatch Repair.** Mismatch repair (MMR) is another important guard against genotoxic stress. In the case of alkylating agents, this pathway plays a critical role in the cytotoxicity mediated by these compounds [70, 83–85]. When alkylation is extensive, MMR is involved in triggering cell death which protects against the mutagenic activity of these agents. For example, MMR recognizes O<sup>6</sup>-mG-T mismatch that occur when AGT is overwhelmed [83]. Unrepaired O<sup>6</sup>-mG is toxic [36]; absence of MMR removes the toxicity of methylating agents indicating that this repair pathway is involved in the mechanism of toxicity [34]. MMR is initiated when the MSH2-MSH6 heterodimer (Mut $\alpha$ ) binds to the mismatch. The MLH1-PMS2 heterodimer then binds to Mut $\alpha$  and triggers removal of the mismatched base. In the case of damaged bases, the mismatch process enters a futile cycle if the adduct is not repaired since polymerases repeatedly insert the wrong base opposite the modified base. This futile cycle can trigger apoptosis [70, 85]. This futile cycle can be thwarted by homologous recombination, a multiprotein pathway that uses the sister chromatid as the template to circumvent replication-halting DNA adducts [85, 86].

The role of mismatch repair in a cell's response to pyridyloxobutyl DNA adducts has not been explored. Preliminary data indicate that O<sup>6</sup>-pobdG may not be a very toxic adduct. Repair of O<sup>6</sup>-pobdG by human AGT in bacteria did not influence the toxicity of the model pyridyloxobutylating

TABLE 3: Levels of  $O^6$ -mG and  $O^6$ -pobG in lung and livers of NNK-treated wild-type and AGT knockout mice<sup>a</sup> [88].

AGT status	pmol adducts/ $\mu$ mol guanine			
	lung		liver	
$O^6$ -mG	24 h	4 weeks	24 h	4 weeks
Wildtype	42 $\pm$ 12	55 $\pm$ 9	17 $\pm$ 11	5.3 $\pm$ 0.7
Knockout	65 $\pm$ 19	110 $\pm$ 20	210 $\pm$ 110	380 $\pm$ 80 <sup>b</sup>
$O^6$ -pobG				
Wildtype	1.7 $\pm$ 0.5	0.8 $\pm$ 0.2	1.4 $\pm$ 0.5	$\leq$ 0.3
Knockout	2.9 $\pm$ 0.6	2.5 $\pm$ 0.3	2.7 $\pm$ 1.2	4.9 $\pm$ 1.4

<sup>a</sup>Mice received either a single dose of NNK (~250 mg/kg) and sacrificed 24 h postinjection or three weekly doses of NNK (~250 mg/kg each week) and sacrificed 1 week after the third treatment. Numbers represent the average of five samples  $\pm$  SD.

<sup>b</sup>Three samples  $\pm$  SD. Two other samples were analyzed and these two animals had  $O^6$ -mG levels of 26 and 31 pmol  $O^6$ -mG/ $\mu$ mol guanine. The liver 7-mG adduct levels for all five animals were similar: 134  $\pm$  17 pmol 7-mG/ $\mu$ mol guanine.

agent, NNKOAc [87]. This observation differs starkly from that observed with methylating agents where the toxicity of a methylating agent is markedly reduced when AGT is expressed [87]. Similar results were observed in CHO cells; AGT expression only minimally reduced the cytotoxicity of NNKOAc while repairing almost 100% of the  $O^6$ -pobdG formed by this pyridyloxobutylating agent [67]. The reduced toxicity of  $O^6$ -pobdG may cause it to more greatly contribute to the overall mutagenic activity of a pyridyloxobutylating agent since cell death protects against the mutagenic activity of DNA alkylating agents.

**5.5. In Vivo Repair.** For both NNK and NNN, the relative distribution of the four pyridyloxobutyl DNA adducts in tissues from exposed rats was significantly different from that observed in DNA treated with a model pyridyloxobutylating agent *in vitro* [56, 57, 59–61]. This difference likely results from the active repair of specific adducts. Further support for this hypothesis is the observed tissue variation in relative adduct distribution [57, 59–61].

One adduct that appears to be well-repaired *in vivo* is  $O^6$ -pobdG [56, 57, 59–61]. The levels of this adduct are very low relative to the other adducts (Tables 1 and 2). In NNK-treated animals, the levels of  $O^6$ -mG were much greater than the levels of  $O^6$ -pobdG and in the range of the other pyridyloxobutyl DNA adducts [58]. This observation suggests that the larger adduct,  $O^6$ -pobdG, is more readily repaired than  $O^6$ -mG *in vivo*. AGT is one pathway clearly responsible for the repair of  $O^6$ -pobdG *in vivo* [54]. However, other repair pathways may also be involved since this adduct does not accumulate in lungs of AGT knockout mice whereas  $O^6$ -mG does (Table 3) [88]. This conclusion is further supported by data in wild-type mice which indicates that AGT is inactivated in mouse lung following exposure to NNK [89].

The most persistent adduct *in vivo* is  $O^2$ -pobdT [56, 57, 59–61]. This adduct is a minor adduct in the absence of repair ( $7$ -pobG >  $O^6$ -pobdG >  $O^2$ -pobdT  $\geq$   $O^2$ -pobC) [46]. This is somewhat surprising since this adduct is repaired by

NER in cell line models [67]. A recent study indicated that NER is reduced in the lungs of NNK-treated mice providing an explanation for the persistence of this adduct *in vivo* [90]. The mechanism of this reduction is unknown.

## 6. Mutagenic Activity of Pyridyloxobutyl DNA Adducts

Pyridyloxobutylating agents are mutagenic in a variety of test systems [67, 87, 91, 92]. However, our knowledge of which pyridyloxobutyl adducts are causing mutations is still rudimentary. Site-specific mutagenesis studies have only been performed for one adduct,  $O^6$ -pobdG [93]. In bacteria, it produces exclusively GC to AT transitional mutations. In human kidney cell line 293 cells, it produces primarily GC to AT transitional mutations with some GC to TA transversions and deletions as well as a number of more complex mutations.

A few studies have begun to link the overall mutagenic activity of pyridyloxobutyl DNA damage to specific adducts through exploring the impact of various DNA repair pathways on the mutagenic properties of the model pyridyloxobutylating agent, NNKOAc. The earliest studies were performed in bacteria. NNKOAc is mutagenic in *Salmonella typhimurium* tester strains TA100, TA1535, and TA98, but not TA102 [92]. Reversion of TA100 and TA1535 requires mutations at a GC base pair and reversion of TA98 requires a frameshift mutation near a CG base pair [94]. TA102 has an AT base pair at the site of reversion [94]. Based on these observations, it was concluded that pyridyloxobutyl DNA adducts formed at GC base pairs were mutagenic, at least in bacteria. However, we cannot rule out that adducts at AT base pairs are not mutagenic in this study since TA102 has an active NER system [94] that could be repairing any mutagenic adducts at AT base pairs. TA100, TA1535, and TA98 lack UvrB and, as a result, do not have an functional NER system [94].

One candidate adduct for the mutagenicity observed in TA100 and TA1535 is  $O^6$ -pobdG. This adduct is poorly

repaired by bacterial AGT [66]. Consistent with its possible role in NNKOAc-induced mutagenicity is the observation that the mutagenic activity of NNKOAc was reduced by roughly 80% in bacteria expressing human AGT [87]. These studies were performed in *S. typhimurium* strain YG7108 which is a derivative of TA1535 that lacks both bacterial AGT genes, *ada* and *ogt* [95]. Since the levels of  $O^6$ -pobdG were reduced in the strain expressing human AGT by about 66% [87], these data are consistent with the hypothesis that  $O^6$ -pobdG is a significant contributor to the mutagenic activity of pyridyloxobutylating agents at GC base pairs. Other contributors may include  $O^2$ -pobC and 7-pobG. However, these two adducts are not substrates for human AGT.

NNKOAc also induced mutations in the *hprt* gene in Chinese hamster ovary (CHO) cells [67]. Analysis of the mutational spectrum indicated that the bulk of the mutations occurred at AT base pairs [67]. Most of the AT mutations were AT to CG transversion mutations. There were also a small portion of AT to TA transversions and AT to GC transitional mutations. Approximately 20% of the mutations were at GC base pairs with the majority of these being GC to AT transitional mutations.

Loss of NER through ERCC-2 mutation results in an increase in mutation frequency induced by NNKOAc in CHO cells [67]. This loss reduced the rate of  $O^2$ -pobdT repair in these cells. In addition, there was a corresponding increase in the frequency of AT to TA mutations relative to the control cell line. Therefore, it is likely that  $O^2$ -pobdT triggers AT to TA mutations. This conclusion is supported by the observation that another  $O^2$ -alkyl-2'-deoxythymidine adduct,  $O^2$ -ethyl-2'-deoxythymidine, also induces AT to TA mutations [96]. Loss of BER through loss of XRCC1 also led to an increase in AT to TA mutations [67], suggesting that this repair pathway is involved in repair of pyridyloxobutyl DNA damage at AT base pairs. One possibility is that  $O^2$ -pobdT is a substrate for BER glycosylases and the result abasic sites are responsible for observed increase in AT to TA mutations observed in the cells lacking BER. This hypothesis is supported by the report that site-specifically incorporated abasic sites primarily induce transversion mutations with AT to TA mutations being more abundant than AT to GC mutations [97].

Expression of human AGT in CHO cells did not significantly impact the mutation frequency of NNKOAc [67]. However, mutations at GC base pairs represented only approximately 20% of the detected mutations. There was a reduction in the GC to AT mutations in these cells but this reduction did not significantly affect the mutation frequency. Since there was almost complete repair of  $O^6$ -pobdG, these data support the hypothesis that  $O^6$ -pobdG is responsible for the GC to AT transitional mutations triggered by pyridyloxobutyl DNA adducts.

*In vivo* studies investigating the mutagenic properties of the pyridyloxobutylating pathway are limited. Mutations were observed in the 12th codon of *K-ras* in lung tumors of A/J mice receiving multiple doses of NNKOAc [98]. Since these mutations were GC to AT transitions and GC to TA transversions, it is likely that  $O^6$ -pobdG is responsible, in part, for these mutations. Both NNK and

NNN have been shown to be mutagenic in target tissues in *lacZ* and *lacI* transgenic mice [88, 99–101]. The resulting transgene mutation spectra have only been reported for NNK [88, 101]. NNK induced an increased rate of GC to AT transitional mutations at non-CpG sites as well as AT to TA transitional mutations and a mixture of transversion mutations (AT to GC, AT to CG, GC to CG, and GC to TA). Since NNK both methylates and pyridyloxobutylates DNA, it is difficult to associate specific mutations with specific adducts. However, it is clear that the mutational spectrum is substantially more complicated than that observed for simple methylating nitrosamines like dimethylnitrosamine, which primarily induces GC to AT transitional mutations at non-CpG sites [102–104].

Collectively, the data presented above indicate that there are several mutagenic DNA adducts formed upon pyridyloxobutylation of DNA. These include  $O^6$ -pobdG and  $O^2$ -pobdT. Other adducts likely contribute as well. Which adducts contribute to the carcinogenic properties of this pathway are likely to depend on the biological system. If mutations at AT base pairs are required to produce proteins with oncogenic function, the formation of  $O^2$ -pobdT and its repair is probably important for tumor initiation by this pathway. On the other hand, if mutations at GC base pairs are important for triggering the carcinogenic process, the formation and persistence of  $O^6$ -pobdG will be linked to tumor formation. For example, GC to AT and GC to TA mutations were observed in the 12th codon of *K-ras* in lung tumors of A/J mice receiving multiple doses of NNKOAc [98]. It is likely that  $O^6$ -pobdG is responsible, in part, for these mutations. Future studies are required to better define the toxicological properties of all pyridyloxobutyl adducts and to determine the repair pathways responsible for protecting against their genotoxic effects. An understanding of these fundamental biochemical issues may help in understanding the individual differences in susceptibility to lung cancer risk associated with tobacco use.

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