Published in final edited form as:
*Chem Res Toxicol*. 2009 August 17; 22(8): 1464–1472. doi:10.1021/tx9001572.

# The influence of repair pathways on the cytotoxicity and mutagenicity induced by the pyridyloxobutylation pathway of tobacco specific nitrosamines

**Li Li**[1,2,5], **Joana Perdigao**[1], **Anthony E. Pegg**[3], **Yanbin Lao**[2,6], **Stephen S. Hecht**[2], **Bruce R. Lindgren**[2], **Joyce T. Reardon**[4], **Aziz Sancar**[4], **Elizabeth V. Wattenberg**[1], and **Lisa A Peterson**[1,2]

[1] Division of Environmental Health Sciences, University of Minnesota, Minneapolis, MN 55455

[2] Masonic Cancer Center, University of Minnesota, Minneapolis, MN 55455

[3] Department of Cellular and Molecular Physiology, The Pennsylvania State University College of Medicine, Hershey, Pennsylvania 17033

[4] Department of Biochemistry and Biophysics, University of North Carolina School of Medicine, Chapel Hill, North Carolina 27599

## Abstract

Tobacco-specific nitrosamines, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) and *N*′-nitrosonornicotine (NNN), are considered to be human carcinogens. Both compounds are metabolized to pyridyloxobutylating intermediates that react with DNA to form adducts such as 7-[4-(3-pyridyl)-4-oxobut-1-yl]-guanine (7-pobG), $O^2$-[4-(3-pyridyl)-4-oxobut-1-yl]-cytosine ($O^2$-pobC), $O^2$-[4-(3-pyridyl)-4-oxobut-1-yl]-2′-deoxythymidine ($O^2$-pobdT), $O^6$-[4-(3-pyridyl)-4-oxobut-1-yl]-2′-deoxyguanosine ($O^6$-pobdG) and 4-hydroxy-1-(3-pyridyl)-1-butanone (HPB)-releasing adducts. The role of specific DNA adducts in the overall genotoxic activity of the pyridyloxobutylation pathway is not known. One adduct, $O^6$-pobdG, is mutagenic. To characterize the mutagenic and cytotoxic properties of pyridyloxobutyl DNA adducts, the impact of DNA repair pathways on the cytotoxic and mutagenic properties of the model pyridyloxobutylating agent, 4-(acetoxymethylnitrosamino)-1-(3-pyridyl)-1-butanone (NNKOAc) was investigated in Chinese hamster ovary (CHO) cell lines proficient or deficient in $O^6$-alkylguanine DNA alkyltransferase (AGT), deficient in both AGT and base excision repair (BER), or deficient in both AGT and nucleotide excision repair (NER). The repair of the four pyridyloxobutyl DNA adducts was determined in the same cell lines via sensitive LC-MS/MS methods. NNKOAc was more cytotoxic in the cell lines lacking AGT, BER and NER repair pathways. It also induced more mutations in the *hprt* gene in the BER and NER deficient cell lines. However, AGT expression did not influence NNKOAc's mutagenicity despite efficient repair of $O^6$-pobdG. Analysis of the *hprt* mutational spectra indicated that NNKOAc primarily caused point mutations at AT base pairs. GC to AT transition mutations were a minor contributor to the overall mutation spectrum, providing a rationale for the observation that AGT does not protect against the overall mutagenic properties of NNKOAc in this model system. The only adduct affected by the absence of effective NER was $O^2$-pobdT.

Correspondence to: Lisa A Peterson.

[5]Current address: Hormel Institute, Campus Mail: 801 16th Ave SE, Austin, MN 55912

[6]Current address: Abbott Laboratories, Drug Metabolism, AP9-1142, 100 Abbott Park Road, Abbott Park, IL 60064

Supporting Information Available: Tables describing the locations and types of mutation in the coding region of the *hprt* gene in mutants derived from NNKOAc-treated CHO cells as well as figures showing the time course of DNA repair in CHO cells and by human excision nuclease.

Slower repair of $O^2$-pobdT in NER deficient cells was associated with increased AT to TA transversion mutations, supporting the hypothesis that these mutations are caused by $O^2$-pobdT. Together, these data support a hypothesis that the pyridyloxobutylation pathway generates multiple mutagenic and toxic adducts.

## Introduction

The tobacco-specific nitrosamines, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) and *N*′-nitrosonornicotine (NNN), are potent carcinogens in laboratory animals, generating tumors at sites comparable to those observed in smokers (1). NNK is a powerful lung carcinogen that induces lung adenocarcinomas in rodents at lifetime doses similar to those experienced by smokers (1). NNN is carcinogenic to the esophagus, nasal cavity and respiratory tract (1). This nitrosamine is present in higher amounts than any other esophageal carcinogen in tobacco smoke. As a result of these and related mechanistic studies, NNK and NNN are considered to be human carcinogens (1,2).

Both NNK and NNN require metabolic activation to form DNA adducts. NNK is metabolized to either a methylating agent or a pyridyloxobutylating agent whereas NNN is metabolized to a pyridyloxobutylating agent (Figure 1). While the contribution of $O^6$-methylguanine ($O^6$-mG) to the carcinogenic properties of NNK has been well studied (1), the role of specific adducts in the mutagenic and carcinogenic properties of the pyridyloxobutylation pathway has not been fully investigated. The majority of the pyridyloxobutyl adducts are unstable to DNA hydrolysis conditions and decompose to release 4-hydroxy-1-(3-pyridyl)-1-butanone (HPB) (3,4). Four pyridyloxobutyl DNA adducts have been recently characterized (Figure 1): 7-[4-(3-pyridyl)-4-oxobut-1-yl]-guanine (7-pobG) (5), $O^2$-[4-(3-pyridyl)-4-oxobut-1-yl]-cytosine ($O^2$-pobC) (6), $O^2$-[4-(3-pyridyl)-4-oxobut-1-yl]-2′-deoxy-thymidine ($O^2$-pobdT) (6), and $O^6$-[4-(3-pyridyl)-4-oxobut-1-yl]-2′-deoxyguanosine ($O^6$-pobdG) (5–7). These adducts have been detected in DNA from NNK- or NNN-treated rodents (8–10).

Several lines of evidence indicate that pyridyloxobutyl DNA adducts likely contribute to the carcinogenic properties of these nitrosamines. First, model compounds that only pyridyloxobutylate DNA are potent mutagens in *Salmonella tymphimurium* (11,12). Second, the model pyridyloxobutylating agent, 4-(acetoxymethylnitrosamino)-1-(3-pyridyl)-1-butanone (NNKOAc), is a lung tumorigen in A/J mice under chronic dosing conditions (13). Third, pyridyloxobutyl DNA adducts accumulate in pulmonary Type II cells and correlate with lung tumor formation in NNK-treated rats (14). Finally, pyridyloxobutyl DNA adducts also build up in normal lung tissues of lung cancer patients (15), indicating that the formation and persistence of these adducts may be important in human lung cancer associated with tobacco use.

The role of specific pyridyloxobutyl DNA adducts in the overall mutagenic and carcinogenic properties of pyridyloxobutylating nitrosamines is unknown. We have demonstrated that $O^6$-pobdG is mutagenic in both bacteria and human cells (16). This adduct produced only GC to AT transition mutations in bacteria whereas in human cells a small number of GC to TA transversions and more complex mutations were also observed. Consistent with the potential importance of this adduct to the mutagenic activity of pyridyloxobutylating agents, GC to AT transitions and GC to TA transversions were observed in the K-*ras* oncogene of tumors induced by NNKOAc in A/J mouse lungs (13). The mutagenic properties of the other pyridyloxobutyl DNA adducts is largely unknown. A preliminary report describing *in vitro* polymerase reactions with $O^2$-pobdT indicates that this adduct likely miscodes during DNA replication (17). However, the mutagenic properties of $O^2$-pobdT have not been studied in a cell-based assay. Together, these data indicate that multiple pyridyloxobutyl DNA adducts likely

contribute to the overall genotoxic properties of the pyridyloxobutylating metabolites of NNK and NNN.

DNA repair pathways protect the cell against the toxic and mutagenic effects of DNA alkylating agents. One repair protein, $O^6$-alkylguanine DNA alkyltransferase (AGT), is important in the repair of $O^6$-pobdG, reducing the mutagenicity of this adduct (16,18). Pyridyloxobutyl DNA adducts are also repaired by nucleotide excision repair (NER) (19), however, the specific adducts removed by this pathway have not been determined. Base excision repair (BER) is a third potential repair pathway for pyridyloxobutyl DNA adducts, however, there is no information on its importance in the overall repair of these DNA adducts.

In this report, we explore the involvement of AGT, NER and BER in protecting a cell from the mutagenic and cytotoxic properties of NNKOAc. In the presence of esterase, NNKOAc decomposes to the same pyridyloxobutylating agent formed upon methyl hydroxylation of NNK or 2′-hydroxylation of NNN (4,20). These studies were performed with Chinese hamster ovary (CHO) cells in which the frequency of point mutations can be readily determined in the X-linked hypoxanthine-guanine phosphoribosyl transferase (*hprt*) loci by growing the treated cells in the presence of 6-thio-guanine. Wild-type cells generate the toxic 6-thioguanine monophosphate and are subsequently killed off. This assay allows for the quantitative measurement of mutation frequency as well as the isolation of mutant colonies for mutation spectrum determination. We determined the cytotoxic and mutagenic properties of NNKOAc in five Chinese hamster ovary (CHO) cell lines - AA8, UV5, EM9, $CHO^{pcDNA3}$ and $CHO^{AGT}$ (Table 1). The parental cell line, AA8, does not express the repair protein AGT (21). UV5 has a mutation in the ERCC-2 gene (XPD) and, as a result, is deficient in NER (22). EM9 lacks XRCC1 and is compromised in BER (23). $CHO^{pcDNA3}$ cells are derived from AA8 cells and are stably transfected with the empty pCMV-neo-*Bam* vector (21). $CHO^{AGT}$ cells are stably transfected with the pCMV-hAGT expressing human AGT (21). These cell lines have been used to determine the importance of DNA repair pathways in protecting cells against the cytotoxic and genotoxic effects of a variety of DNA damaging agents (21,24–30). We measured the influence of the repair proteins on the mutational spectrum of NNKOAc as well as the formation and repair of the known pyridyloxobutyl DNA adducts, 7-pobG, $O^6$-pobdG, $O^2$-pobC and $O^2$-pobdT. Collectively, these studies shed new insights into the mutagenic and toxic properties of pyridyloxobutyl DNA adducts.

## Materials and Methods

Caution: *NNKOAc is toxic and mutagenic in cells and is carcinogenic in laboratory animals. It should be handled with proper safety equipment and precautions.*

### Chemicals

NNKOAc, [pyridine-$^2H_4$]$O^2$-[4-(3-pyridyl)-4-oxobut-1-yl]cytosine, [pyridine-$^2H_4$]7-[4-(3-pyridyl)-4-oxobut-1-yl]guanine, [1,2,2-$^2H_3$]$O^6$-[oxo-4-(3-pyridyl)butyl]-2′-deoxyguanosine and [pyridine-$^2H_4$]$O^2$-[4-(3-pyridyl)-4-oxobut-1-yl]thymidine were synthesized as previously described (20,31,32). Except where indicated, reagents were from Sigma (St. Louis, MO).

### Cell Lines

CHO cell lines AA8 (wild-type), UV5 (XPD defects) and EM9 (XRCC1 defects) were obtained from American Type Culture Collection (Manassas, Virginia). These experiments were also performed with AA8 cells stably transfected with the pCMV-neo-*Bam* vector without an inserted cDNA sequence ($CHO^{pcDNA3}$) or the same plasmid expressing human AGT ($CHO^{AGT}$) (21). Western blot analysis confirmed the expression of AGT in $CHO^{AGT}$ cells (21). The AGT activity in the cells was approximately 450 fmol/mg protein (21). The cell lines

were maintained in Minimum Essential Medium Alpha Modification (αMEM) supplemented with 10% fetal bovine serum (Invitrogen, Carlsbad, CA) and 1% penicillin/streptomycin at 37° C in a humidified 5% $CO_2$ incubator. Pre-existing *hprt* mutants were eliminated by growing the cells in complete medium supplemented with 100 μM hypoxanthine, 0.4 μM aminopterin and 16 μM thymidine as previously described (25).

## Survival and mutant frequency

Cytotoxicity and mutation frequency at the *hprt* locus was determined according to published methods (33,34). Six-well plates were seeded with $2 \times 10^5$ exponentially growing cells 18–24 h prior to treatment. The cells were exposed to 0–48 μM NNKOAc for 1 h. Each treatment was performed in duplicate. The cells were washed with PBS and grown in fresh medium for 16–24 h. The cells were then harvested, counted, and replated for measurement of colony forming ability and mutant frequency measurements. Each experiment was repeated a minimum of three times.

Colony forming ability was determined by plating 100 cells from each treatment into 6-well plates (33). Two wells were plated per treatment. The plating efficiencies were similar for all five cell lines. After 6–7 days growth in normal medium, colonies were stained with 0.05% crystal violet and were counted with an aCOLyte colony counter (Synbiosis, Frederick, MD). Cell survival was normalized to the number of colonies from untreated cells.

For the mutagenesis assay, each treated culture was plated into 60 mm dishes and grown in normal medium, with subculturing, to allow phenotypic expression of induced mutants. After 7 days, the cells were harvested, counted and plated for determination of cloning efficiency and mutant frequency. The cloning efficiency was determined by seeding 200 cells into 60 mm dishes in normal medium. Colonies were stained with crystal violet after 6–7 days growth and counted. The mutant frequency was determined by plating 100,000 cells into 100 mm dishes in medium containing 5 μg/ml 6-thioguanine to select for mutant colonies. Ten dishes per treatment were plated. Colonies were stained after 8 days of growth in the selection medium and counted with an aCOLyte colony counter. The mutant frequency was expressed as the 6-thioguanine resistant colonies per 100,000 viable cells.

The effect of cell type on cytotoxicity was determined using analysis of variance at each concentration of NNKOAc. Since the F-test for the simple effect was significant, pair wise comparisons of UV5 and EM9 with AA8 were conducted. At each step of the analysis, the level of significance was 0.05; the calculated *p*-values were unadjusted for multiple comparisons. The two-sample t-test was employed to compare mutant frequency between cell lines at each concentration of NNKOAc.

## Mutation spectrum in hprt gene

**Cell treatment—**CHO cells ($1\times10^6$) were seeded into 100 mm dishes 16–24 h prior to treatment. The cells were exposed to 24 μM NNKOAc for 1 h, washed once with PBS and further grown in drug-free fresh medium for 16–24 h. The cells were then harvested and split into 10 × T25 flasks. The cells were grown in normal complete medium for 7 days to allow the phenotypic expression of mutants, with two subcultures during the 7 day period to avoid confluence. Cells were then harvested, counted, and plated into 100 mm dishes (100,000 cells/dish) in medium containing 5 μg/mL 6-thioguanine (selection medium). Two plates were generated from each T25 flask. After 9 days growth in the selection medium, 2 mutant colonies from each plate were picked and transferred to 24 well plates for continued growth in selection medium. The cells in the 24-well plates were harvested and transferred into 100 mm dishes respectively 3 days later, and continued growth in selection medium. Once the cells were 80–

90% confluent, they were harvested, counted, and aliquoted into 50,000 cell portions for RT-PCR analysis.

A total of 36 (EM9), 39 (UV5, AA8), 57 ($CHO^{pcDNA3}$) or 69 ($CHO^{AGT}$) mutant colonies were picked and expanded for molecular characterization. Control and mutant *hprt* mRNA was reversed transcribed and amplified for sequence analysis. If two clones from the same flask displayed identical mutations, one of these clones was removed from our analysis since they were likely sibling clones. In samples where the PCR product was absent, truncated or produced multiple amplicons, PCR analysis of the nine *hprt* exons was performed with genomic DNA (25,26). These latter studies allowed us to determine if the irregularities in the initial reverse transcript PCR reactions resulted from large deletions or mutations resulting in splicing defects.

**PCR analysis—**cDNA was prepared from lysates of mutant clones ($5 \times 10^4$ cells) with a Cells-to-cDNA II Kit (Ambion, Austin, TX) according to manufacturer instructions. The cDNA was amplified using *hprt*-specific primers and Platinum Taq DNA Polymerase High Fidelity (Invitrogen). In a first round of PCR, a 789 bp product was generated using primers 1 (*hprt* positions 23–42: 5′-CTCGGCGCCTCCTCTGCGGG) and 2 (*hprt* positions 792–811: 5′-GGTAATTTTACTGGGAAC). The PCR cycling conditions were as follows: initial melting step (94 °C, 2 min), 35 cycles of denaturation (94 °C, 40 sec). annealing (55 °C, 40 sec) and extension (72 °C, 1 min) followed by a final extension step at 72 °C for 7 min. PCR products were analyzed on an agarose gel and if a band at 789 bp was present, then the PCR band was purified for sequencing using the QIAquick PCR Purification Kit (Qiagen, Valencia, CA). If the 789 bp band was absent or weak, then a nested PCR was performed with primers 3 (*hprt* positions 47–66: 5′-CTCCTCACACCGCTCTTCGC) and 4 (*hprt* positions 764–783: 5′-CTCCTCGTGTTTGCAGATTC). The PCR reactions were performed as described above with the exception that the annealing was performed at 60 °C. The corresponding 737 bp product was excised from the gel and purified using the QIAquick Gel Extraction Kit (Qiagen). All PCR products were sequenced at the Biomedical Genomics Center, University of Minnesota.

**Genomic PCR—**In samples where the cDNA PCR product was absent, truncated or produced multiple amplicons, the *hprt* exons 1–9 were amplified with genomic DNA as the template according to a published method (26). Genomic DNA was isolated from $2–5 \times 10^6$ cells with DNeasy Blood and Tissue isolation kit (Qiagen) according to the manufacturer instructions. Multiplex PCR reactions were performed exactly as described (26). The reaction products were visualized on 1.3% agarose gels. These PCR products were not sequenced.

Chi-square analysis and Fisher's exact test were used for the comparisons of the mutational spectra between cell lines. The *p*-values were unadjusted for multiple comparisons.

## DNA adduct analysis

Cells (~$1.5 \times 10^6$) were seeded into 150 mm dishes. Three days later, the cells were exposed to 0–32 μM NNKOAc for 1 h. Each condition was performed in triplicate. At 0–48 h, cells were harvested and the contents of three plates were pooled for DNA isolation using Nucleobond AX-G DNA isolation kits (Clontech, Palo Alto, CA). Adduct levels were determined with LC-MS/MS according to previously published methods (31,32,35). Levels of 7-pobG, $O^6$-pobdG, $O^2$-pobC and $O^2$-pobdT were measured in enzyme hydrolysates and expressed relative to the 2′-deoxyguanosine concentration in the hydrolysates (31,32). In addition, levels of the nucleobase, $O^6$-[4-(3-pyridyl)-4-oxobut-1-yl]-guanine ($O^6$-pobG), were determined in 0.1 N HCl hydrolysates and expressed relative to the guanine concentration in the samples (35).

### In vitro NER repair assay

**Preparation of DNA substrates—**A 144-bp duplex with a centrally located $O^6$-pobdG adduct was prepared by annealing and ligation of six complementary and partially overlapping oligomers as described (36). Control substrates, a 144-bp undamaged (UM) duplex and a 136-bp duplex with a T[6-4]T, were prepared in a similar manner. Oligonucleotides employed to build the 144-bp duplex were obtained from Operon Technologies (Alameda, CA) or were prepared in house (37,38). The sequence of the $O^6$-pobdG containing oligonucleotide was AATAGTAGCTG*GAGGC, where *G was $O^6$-pobdG. This oliognucleotide was 5′ end-labeled with $\gamma$-$^{32}$P-ATP and used to generate a 144-bp duplex with a radiolabel positioned 11 phosphodiester bonds 5′ to the damage. The dual incision event (excision) bracketing the lesion on the radiolabeled strand results in release of 24–32-mers containing both the damage and the radiolabel; this repair signal is detected and quantified as described below. The 136-bp duplex with T[6-4]T was prepared in a similar manner using an 8-mer containing the T[6-4]T substrate (36).

**Analysis of Nucleotide Excision Repair—**The six factors of the human excision nuclease, RPA, XPA, XPC•hR23B, TFIIH, XPF•ERCC1, and XPG, were purified and stored as described (36). Excision assays were performed as described (36) with 10 fmol substrate and the indicated amounts of repair factors in a 12.5 μL reaction. For kinetic assays, 20 fmol substrate in a 25 μL reaction volume was used and aliquots were removed at the indicated time points. Reactions were terminated by proteinase K treatment for 15 min at 55–60° C followed by phenol and phenol:chloroform extractions. DNA was recovered after ethanol precipitation, resuspended in formamide/dye mixture, and resolved in 10% sequencing gels. Excision was visualized by autoradiography and percentage excision was quantified using a PhosphorImager (Storm 860) and ImageQuant program (GE Healthcare, Piscataway, NJ). Excision levels for each reaction were determined as a percentage of radiolabel in the excision product (24–32 nt) region of the gel relative to the total radiolabel in the substrate migrating at 136 or 144 nt plus excision products in that lane.

## Results

### Cytotoxicity and mutagenesis

The cytotoxicity and mutagenic activity of NNKOAc were determined in five CHO cell lines - AA8, UV5, EM9, CHO$^{pcDNA3}$ and CHO$^{AGT}$. All five cell lines were treated with increasing concentrations of NNKOAc for 1 h and then plated for assessment of cytotoxicity or mutagenesis. Cell survival was determined via a colony forming assay. As shown in Figure 2A, UV5 and EM9 cells were more sensitive to the cytotoxic effects of NNKOAc than the parental cell line, AA8. In addition, NNKOAc was more toxic in CHO$^{pcDNA3}$ cells relative to CHO$^{AGT}$ cells (Figure 2C).

To determine the ability of the various repair pathways to influence the frequency of point mutations, the mutation frequency in the *hprt* gene was measured by selecting mutants with 6-thio-guanine. NNKOAc induced *hprt* mutations in the AA8 cells in a dose dependent fashion (Figure 2B). The frequency of mutations induced by NNKOAc was elevated in the UV5 cells at the two lowest concentrations and in EM9 at the two highest concentration (Figure 2B). The expression of hAGT did not influence the mutagenic activity of NNKOAc (Figure 2D).

The mutation spectra were characterized in NNKOAc-treated cells by sequencing the *hprt* cDNA isolated from 6-thioguanine-resistant mutants. In samples where the PCR product was absent, truncated or produced multiple amplicons, PCR analysis of the nine *hprt* exons was performed with genomic DNA (25,26). These latter studies allowed us to determine if the irregularities in the initial reverse transcript PCR reactions resulted from large deletions or

mutations resulting in splicing defects. Results of the sequencing studies are displayed in Supplemental Tables 1–5 and summarized in Supplemental Table 6. The vast majority of *hprt* mutations induced by NNKOAc were point mutations (Supplemental Table 6). The spectrum of NNKOAc-induced mutations is significantly different than that reported for spontaneous mutations in these cell lines ($p$-value < 0.001, Table 2) (26,39). Most of these NNKOAc-induced point mutations (≥ 70%) occurred at AT base pairs, with tranversions as the dominant change (Table 2). The majority of these transversions were AT to CG mutations.

Alterations in the DNA repair status of the cell line had modest impact on the distribution of mutations (Table 2). For example, there were significant differences in the extent of AT to CG and AT to TA mutations between the cell lines ($p < 0.05$). The distribution of NNKOAc-derived mutations was much broader in the NER deficient cells (UV5) with fewer AT to CG mutations and more AT to TA mutations than the cell lines with functional NER (AA8). There were also more AT to TA mutations in the BER deficient EM9 cell line. While there is a trend towards fewer GC to AT transition mutations in $CHO^{AGT}$ relative to the other cell lines, this trend was not statistically significant.

## Pyridyloxobutyl DNA adducts analysis

To determine which adducts were differentially repaired in each cell line, levels of $O^2$-pobC, 7-pobG, $O^2$-pobdT and $O^6$-pobdG were measured using established LC-MS/MS methods (31,32). Preliminary studies to investigate cell line differences in initial adduct levels were performed with a 1 h exposure of UV5, AA8, $CHO^{AGT}$ and $CHO^{pcDNA3}$ cells to 32 μM NNKOAc. All cell lines had similar initial levels of each pyridyloxobutyl DNA adducts, except $O^6$-pobdG (Figure 3). The levels of this adduct were significantly lower in $CHO^{AGT}$ cells, consistent with previous reports that $O^6$-pobdG is readily repaired by AGT (7,18,40).

The time course of adduct removal was determined by exposing the CHO cell lines to 8 μM NNKOAc and then monitoring the disappearance of the four pyridyloxobutyl DNA adducts at 0, 4, 24 and 48 h after exposure. This concentration was chosen since it was only slightly toxic, killing less than 20–25% of cells in each cell line (Figures 2A and 2C). Also, we wanted to ensure that there were functioning repair systems at non-saturating adduct levels. As expected, there was no significant difference in adduct levels between any of the five cell lines immediately after the 1 h exposure. The only exception was $O^6$-pobdG; it was significantly lower in $CHO^{AGT}$ cells. When $CHO^{pcDNA3}$ and $CHO^{AGT}$ cells were compared, there was no significant difference in the rate of repair of $O^2$-pobC, 7-pobG or $O^2$-pobdT (data not shown). $O^6$-pobdG was completely repaired in $CHO^{AGT}$ cells by 24 h whereas it persisted in $CHO^{pcDNA3}$ cells (data not shown). The rates of removal of $O^2$-pobC and 7-pobG were similar in AA8, UV5 and EM9 cells (Figure 4). In contrast, $O^2$-pobdT was more persistent in UV5 cells which lack functional NER (Figure 4). There was no apparent difference in the removal of $O^6$-pobdG in AA8, UV5 or EM9 cells. Since there was significant noise in the measurement of $O^6$-pobdG adduct levels in these cell lines, DNA samples from AA8 and UV5 cells were also subjected to acid hydrolysis to measure the levels of the nucleobase, $O^6$-pobG (35). When this method of analysis was employed, the data was cleaner and confirmed there was no significant difference in the rate of disappearance of $O^6$-pobG between cell lines (Figure 4).

## *In vitro* NER-mediated repair of $O^6$-pobdG

Since other large $O^6$-alkylguanine adducts are substrates for NER (41), it was somewhat surprising that the repair of $O^6$-pobdG was unaffected by the loss of ERCC2 activity in UV5 cells. To further explore this possibility, the ability of NER to repair $O^6$-pobdG was determined *in vitro*. A reconstituted human excision nuclease RFI-VI (repair factors RPA, XPA, XPC, TFIIH, XPG and XPF-ERCC1) was incubated with a $^{32}$P-labeled 144-bp duplex containing a centrally located $O^6$-pobdG adduct and the resulting excision products were separated by gel

electrophoresis. Analysis of these gels indicated that a small amount of the $O^6$-pobdG had been excised (Figure 5). Levels of excision for $O^6$-pobdG (1.1%) were slightly greater than that observed for unmodified DNA (0.8%) but significantly less than the levels observed for a T [6-4]T photoproduct (32.7%). This excision proceeded in a time-dependent fashion (Supplemental Figure 1). These data confirm the cell-based results by demonstrating that $O^6$-pobdG is a poor substrate for repair by the NER machinery.

## Discussion

The involvement of specific pyridyloxobutyl DNA adducts in the overall cytotoxic and mutagenic properties of a pyridyloxobutylating agent has not been previously investigated. Therefore, we determined the cytotoxic and mutagenic properties of the model pyridyloxobutylating agent, NNKOAc, in CHO cell lines that vary in DNA repair capacity. These effects were then compared to the lifetime of specific pyridyloxobutyl DNA adducts in the same cell lines to determine which of these adducts is responsible for the toxic and mutagenic activities of NNKOAc.

NNKOAc is toxic and mutagenic in CHO cells (Figure 2). The spectrum of mutations induced by NNKOAc in the *hprt* gene is significantly different from that observed in untreated cells (Table 2). NNKOAc caused mutations primarily at AT base pairs, regardless of repair background whereas the spontaneous mutations occurred primarily at GC base pairs (Table 2). In AA8 cells, AT to CG transversion mutations were the major type of base substitution in contrast to the GC to TA transversions that dominate the spontaneous mutation spectrum (Table 2). *In vivo* mutagenesis studies with NNK in the Muta™ Mouse and Big Bl™ mouse model systems indicated that AT to TA and/or AT to CG mutations are major mutations observed in the transgene in these animals (35,42). Our data suggest that these mutations may be caused by pyridyloxobutyl DNA adducts.

It is possible that $O^2$-pobdT is responsible for the mutations observed at AT base pairs. *In vitro* polymerase reactions with $O^2$-pobdT indicate that all four nucleotides can be incorporated opposite this modified base (17). However, we cannot rule out the possible involvement of less stable adenine or thymine adducts for the induction of mutations at AT base pairs since HPB-releasing adducts are a significant contributor to the overall DNA damage formed by pyridyloxobutylating agents (43) and these unstable adducts could form at AT base pairs. Mutations at GC base pairs represented approximately 30% of the NNKOAc-induced *hprt* mutations. The majority of these mutations are GC to AT transition mutations. Therefore, only 17–25% of the mutations were likely to be caused by $O^6$-pobdG which has been shown to induce primarily GC to AT mutations in human cell lines (16).

### The influence of NER on NNKOAc-induced cytotoxicity and mutagenicity

The cytotoxic and mutagenic effects of NNKOAc were roughly doubled in the NER-compromised cell line, UV5, relative to AA8 cells. These results are consistent with the report that NNKOAc was modestly more cytotoxic in XPA (2.3 fold) or XPC (1.5 fold) deficient human lymphoid cell lines relative to NER competent cell lines (19). The impact of XPD deficiency on NNKOAc's mutagenic effects in UV5 cells is comparable to that observed for structurally related alkylating agents, *n*-butylnitrosourea and ethylnitrosourea (24,29). *n*-Butylnitrosourea was 1.4-fold more cytotoxic and 40% more mutagenic in UV5 cells as compared with AA8 cells (24). The mutagenic activity of the ethylating agent, ethylnitrosourea increased by ~50% in UV5 cells relative to AA8 while the cytotoxicity was unaffected (29).

It has been reported that pyridyloxobutyl DNA adducts are repaired by NER as judged by the ATP-dependent incorporation of [$\alpha^{32}$P]dTTP into plasmid DNA upon incubation with extracts from NER competent and deficient human lymphoid cell lines; cell extracts from XPA and

XPC-deficient cell lines were less active in their ability to repair pyridyloxobutylated DNA (19). Consistent with that report, our observations using the UV5 cell line suggest that NER may be involved in the repair of cytotoxic and mutagenic pyridyloxobutyl DNA adducts.

The only pyridyloxobutyl adduct that was impacted by the loss of XPD activity in UV5 cells was $O^2$-pobdT (Figure 4). Since the reduced repair of this adduct is accompanied by increased cytotoxicity and mutagenicity, we propose that $O^2$-pobdT is both a cytotoxic and mutagenic pyridyloxobutyl DNA adduct. This adduct is the dominant pyridyloxobutyl adduct detected in lung or liver DNA isolated from NNK or NNN-treated rats (9,10). Therefore, it is likely that this adduct contributes to the carcinogenic properties of pyridyloxobutylating nitrosamines.

There was an increase in the frequency of AT to TA mutations in UV5 relative to AA8 (Table 3), suggesting that NER is involved in the repair of pyridyloxobutyl DNA adducts that trigger this transversion mutation. Since $O^2$-pobdT is poorly repaired in UV5 cells, we hypothesize that this adduct may trigger AT to TA mutations. Consistent with this hypothesis, structurally related $O^2$-ethylthymine triggers AT to TA mutations in site-specific mutagenesis studies (44). Site-specific mutagenesis studies with $O^2$-pobdT are required to directly demonstrate the genotoxicity of this adduct.

Surprisingly, there was no significant difference in the rate of repair of $O^6$-pobdG between parental AA8 and NER-deficient UV5 cells. Consistent with the results in CHO cells, $O^6$-pobdG is a poor substrate for human NER *in vitro*, although it is a better substrate for this repair pathway than $O^6$-methylguanine (data not shown). These findings differ with those reported for other $O^6$-alkylguanine adducts. For example, the bulky $O^6$-butylguanine is thought to be repaired by nucleotide excision repair (41). It is possible that $O^6$-pobdG is slowly repaired via NER since it does not accumulate in lung DNA in NNK-treated mice lacking AGT (35). This observation is in contrast to the accumulation of $O^6$-mG in the lung DNA of the same animals.

### The influence of BER on NNKOAc-induced cytotoxicity and mutagenicity

The cytotoxic and mutagenic effects of NNKOAc were roughly doubled in EM9 cells relative to AA8 cells (Figure 2). EM9 cells lack functional XRCC1 which is an important scaffold protein that facilitates numerous steps in BER repair (45). Knockout of this protein sensitizes cells to the DNA damaging effects of a variety of alkylating agents (23). While our studies provide no information as to which pyridyloxobutyl DNA adducts are being repaired by BER, they suggest that DNA pyridyloxobutylation results in the formation of DNA damage that requires XRCC1 activity in order for the cell to escape the cytotoxic and mutagenic effects of this damage.

There are two potential pathways for pyridyloxobutyl DNA adducts to generate DNA damage that could enter into BER. First, there may be specific pyridyloxobutyl DNA adducts that are substrates for BER glycosylases. There is evidence that structurally related $O^6$-butylguanine may be repaired by base excision repair *in vivo* (46). Therefore, it is possible that one or more of the pyridyloxobutyl DNA adducts are substrates for this group of repair proteins. Second, pyridyloxobutylation of DNA generates abasic sites since both 7-pobG and $O^2$-pobC spontaneously depurinate/depyrimidate from DNA. These resultant abasic sites are expected to be repaired by the BER machinery.

There was an increase in the mutation frequency of AT to TA mutations in EM9 cells relative to AA8 cells (Table 3). Studies performed with site-specific abasic sites indicate that this type of lesion triggers predominantly transversions with AT to TA mutations more prevalent than AT to GC mutations (47). Therefore, we postulate that abasic sites generated either by glycosylase catalyzed removal of a pyridyloxobutylated base or by spontaneous depurination/depyrimidation can contribute to the mutagenic activity of pyridyloxobuylating agents.

### The influence of AGT on NNKOAc-induced cytotoxicity and mutagenicity

The expression of human AGT in the CHO cell lines had a slight impact on the cytotoxicity of NNKOAc. These data suggest that $O^6$-pobdG may be toxic to mammalian cells. This adduct was not toxic in bacteria (18). Surprisingly, there was no effect of AGT expression on the genotoxicity of NNKOAc. These observations contrast dramatically with those reported for methylating agents (48,49) and with results obtained for NNKOAc in bacteria expressing human AGT (18). Analysis for DNA adducts demonstrated that $O^6$-pobdG was almost completely repaired in $CHO^{AGT}$ cells (Figure 3), confirming that this adduct is rapidly repaired by human AGT (18,40). Therefore, it appears that $O^6$-pobdG is not a major contributor to the mutagenic activity of NNKOAc in the *hprt* gene of CHO cells.

The spectrum of NNKOAc-induced *hprt* mutations provides some clues to why $O^6$-pobdG does not contribute significantly to the mutational activity of NNKOAc in the CHO model system. The majority of the mutations (83%) in the wild-type cell line, $CHO^{pcDNA3}$, occur at AT base pairs, not GC base pairs. Therefore, it is likely that the repair of mutagenic $O^6$-pobdG adducts does not affect the overall mutation frequency since they contribute only to a small percentage of the overall mutations. However, there is a decrease in the frequency of transition mutations at GC base pairs in $CHO^{AGT}$ cells (Table 3), consistent with AGT mediated repair of $O^6$-pobdG. Our previous studies in *Salmonella typhimurium* demonstrated that human AGT protects against the mutagenic activity of NNKOAc (18). This particular model system requires mutagenesis at GC base pairs. Therefore, the ability of AGT to protect against the mutagenic effects of a pyridyloxobutylating agent will be dependent on the mutations required to alter the phenotype of the protein. Consistently, NNKOAc induced GC to AT transitions as well as GC to TA transversions in the twelfth codon of the K-*ras* oncogene of tumors in A/J mice (13). $O^6$-pobdG is likely responsible for the GC to AT mutations observed in this system.

### Summary

Overall, BER and NER are important pathways in the protection of cells against the cytotoxic and genotoxic effects of the pyridyloxobutylating pathways of tobacco-specific nitrosamines. It is likely that genetic variations in these repair pathways will influence the effectiveness of this protection and contribute to an individual's sensitivity to the carcinogenic properties of the tobacco-specific carcinogens NNK and NNN. Our data are consistent with the hypothesis that multiple adducts contribute to the overall mutagenic properties of this pathway. This study suggests that $O^2$-pobdT is a cytotoxic and mutagenic adduct and previous studies demonstrated that $O^6$-pobdG is also a mutagenic adduct (16,18). Which adducts are crucial to the carcinogenic properties of this pathway are likely to vary depending on the system. If mutations at AT base pairs selectively produce proteins with oncogenic function, the formation of $O^2$-pobdT and its repair by NER is probably important for tumor initiation by this pathway. On the other hand, if mutations at GC base pairs are important for initiating tumors, the formation $O^6$-pobdG and its repair by AGT will be important. Future studies will investigate the importance of these repair pathways to protect against the genotoxic effects of pyridyloxobutyl DNA adducts in humans.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgments

The authors would like to thank Dr. Peter Villalta for his assistance with the mass spectral analyses, Choua Vu for the synthesis of NNKOAc and Bob Carlson for his help with the figures. The Analytical Biochemistry Shared Resource is supported in part by Cancer Center Support Grant CA-77598. The research was supported by the following National Institutes of Health grants: CA-115309 (LAP), GM-32833 (AS), CA-018137 (AEP) and CA-081301 (SSH).

## Abbreviations

| | |
|---|---|
| ***O*$^6$-mG** | $O^6$-methylguanine |
| **7-pobG** | 7-[4-(3-pyridyl)-4-oxobut-1-yl]-guanine |
| ***O*$^2$-pobC** | $O^2$-[4-(3-pyridyl)-4-oxobut-1-yl]-cytosine |
| ***O*$^2$-pobdT** | $O^2$-[4-(3-pyridyl)-4-oxobut-1-yl]-2′-deoxythymidine |
| ***O*$^6$-pobdG** | $O^6$-[4-(3-pyridyl)-4-oxobut-1-yl]-2′-deoxyguanosine |
| ***O*$^6$-pobG** | $O^6$-[4-(3-pyridyl)-4-oxobut-1-yl]-guanine |
| **AGT** | $O^6$-alkylguanine DNA alkyltransferase |
| **BER** | base excision repair |
| **CHO** | Chinese hamster ovary |
| **HPB** | 4-hydroxy-1-(3-pyridyl)-1-butanone |
| ***hprt*** | hypoxanthine-guanine phosphoribosyl transferase |
| **NER** | nucleotide excision repair |
| **NNK** | 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone |
| **NNKOAc** | 4-(acetoxymethylnitrosamino)-1-(3-pyridyl)-1-butanone |
| **NNN** | *N*-nitrosonornicotine |

## Reference List


1. Hecht SS. Biochemistry, biology, and carcinogenicity of tobacco-specific *N*-nitrosamines. Chem Res Toxicol 1998;11:560–603.
2. Hecht SS. Tobacco smoke carcinogens and lung cancer. J Natl Cancer Instit 1999;91:1194–1210.
3. Hecht SS, Spratt TE, Trushin N. Evidence for 4-(3-pyridyl)-4-oxobutylation of DNA in F344 rats treated with the tobacco specific nitrosamines 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone and *N*′-nitrosonornicotine. Carcinogenesis 1988;9:161–165. [PubMed: 3335041]
4. Peterson LA, Hecht SS. $O^6$-Methylguanine is a critical determinant of 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone tumorigenesis in A/J mouse lung. Cancer Res 1991;51:5557–5564. [PubMed: 1913675]
5. Wang M, Cheng G, Sturla SJ, McIntee EJ, Villalta PW, Upadhyaya P, Hecht SS. Identification of adducts formed by pyridyloxobutylation of deoxyguanosine and DNA by 4-(acetoxymethylnitrosamino)-1-(3-pyridyl)-1-butanone, a chemically activated form of tobacco specific carcinogens. Chem Res Toxicol 2003;16:616–626. [PubMed: 12755591]
6. Hecht SS, Villalta PW, Sturla SJ, Cheng G, Yu N, Upadhyaya P, Wang M. Identification of $O^2$-substituted pyrimidine adducts formed in reactions of 4-(acetoxymethylnitrosamino)- 1-(3-pyridyl)-1-butanone and 4-(acetoxymethylnitrosamino)-1-(3-pyridyl)-1-butanol with DNA. Chem Res Toxicol 2004;17:588–597. [PubMed: 15144215]
7. Wang L, Spratt TE, Liu XK, Hecht SS, Pegg AE, Peterson LA. Pyridyloxobutyl adduct $O^6$-[4-oxo-4-(3-pyridyl)butyl]guanine is present in 4-(acetoxymethylnitrosamino)-1-(3-pyridyl)-1-butanone-treated DNA and is a substrate for $O^6$-alkylguanine-DNA alkyltransferase. Chem Res Toxicol 1997;10:562–567. [PubMed: 9168254]
8. Thomson NM, Kenney PM, Peterson LA. The pyridyloxobutyl DNA adduct, $O^6$-[4-oxo-4-(3-pyridyl) butyl]guanine, is detected in tissues from 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone-treated A/J mice. Chem Res Toxicol 2003;16:1–6. [PubMed: 12693024]

9. Lao Y, Yu N, Kassie F, Villalta PW, Hecht SS. Formation and accumulation of pyridyloxobutyl DNA adducts in F344 rats chronically treated with 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone and enantiomers of its metabolite, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol. Chem Res Toxicol 2007;20:235–245. [PubMed: 17305407]
10. Lao Y, Yu N, Kassie F, Villalta PW, Hecht SS. Analysis of pyridyloxobutyl DNA adducts in F344 rats chronically treated with (R)- and (S)-*N*′-nitrosonornicotine. Chem Res Toxicol 2007;20:246–256. [PubMed: 17305408]
11. Foiles PG, Peterson LA, Miglietta LM, Ronai Z. Analysis of mutagenic activity and ability to induce replication of polyoma DNA sequences by different model compounds of the carcinogenic tobacco-specific nitrosamine 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone. Mutat Res 1992;279:91–101. [PubMed: 1375343]
12. Hecht SS, Lin D, Castonguay A. Effects of α-deuterium substitution on the mutagenicity of 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK). Carcinogenesis 1983;4:305–310. [PubMed: 6339096]
13. Ronai ZA, Gradia S, Peterson LA, Hecht SS. G to A transitions and G to T transversions in codon 12 of the Ki-*ras* oncogene isolated from mouse lung tumors induced by 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) and related DNA methylating and pyridyloxobutylating agents. Carcinogenesis 1993;14:2419–2422. [PubMed: 7902220]
14. Staretz ME, Foiles PG, Miglietta LM, Hecht SS. Evidence for an important role of DNA pyridyloxobutylation in rat lung carcinogenesis by 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone: effects of dose and phenethyl isothiocyanate. Cancer Res 1997;57:259–266. [PubMed: 9000565]
15. Holzle D, Schlobe D, Tricker AR, Richter E. Mass spectrometric analysis of 4-hydroxy-1-(3-pyridyl)-1-butanone-releasing DNA adducts in human lung. Toxicology 2007;232:277–285. [PubMed: 17321028]
16. Pauly GT, Peterson LA, Moschel RC. Mutagenesis by $O^6$-[4-oxo-4-(3-pyridyl)butyl]guanine in *Esherichi coli* and human cells. Chem Res Toxicol 2002;15:165–169. [PubMed: 11849042]
17. Sharma AK, Gowdahalli K, Krzeminski J, Desai D, Lin JM, Gowda ASP, Spratt TE, Amin S. Facile synthesis and mutagenicity studies of $O^2$-[4-(3-pyridyl)-4-oxobut-1-yl]thymidine, the major adduct formed by tobacco-specific nitrosamine 4-methylnitrosamino-1-(3-pyridyl)-1-butanone (NNK) in vivo, and its site-specifically adduct oligodeoxynucleotide. Proc Am Assoc Cancer Res 2008;49:530.
18. Mijal RS, Loktionova NA, Vu CC, Pegg AE, Peterson LA. $O^6$-Pyridyloxobutylguanine adducts contribute to the mutagenic properties of pyridyloxobutylating agents. Chem Res Toxicol 2005;18:1619–1625. [PubMed: 16533027]
19. Brown PJ, Bedard LL, Massey TE. Repair of 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone-induced DNA pyridyloxobutylation by nucleotide excision repair. Cancer Lett 2008;260:48–55. [PubMed: 18037231]
20. Spratt TE, Peterson LA, Confer WL, Hecht SS. Solvolysis of model compounds for α-hydroxylation of *N*′-nitrosonornicotine and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone: Evidence for a cyclic oxonium ion intermediate in the alkylation of nucleophiles. Chem Res Toxicol 1990;3:350–356. [PubMed: 2133084]
21. Loktionova NA, Pegg AE. Point mutations in human $O^6$-alkylguanine-DNA alkyltransferase prevent the sensitization of $O^6$-benzylguanine to killing by N,N′-*bis*(2-chloroethyl)-*N*-nitrosourea. Cancer Res 1996;56:1578. [PubMed: 8603405]
22. Thompson LH, Brookman KW, Dillehay LE, Mooney CL, Carrano AV. Hypersensitivity to mutation and sister-chromatid-exchange induction in CHO cell mutants defective in incising DNA containing UV lesions. Somatic Cell Genet 1982;8:759–773. [PubMed: 7163954]
23. Thompson LH, Brookman KW, Dillehay LE, Carrano AV, Mazrimas JA, Mooney CL, Minkler JL. A CHO-cell strain having hypersensitivity to mutagens, a defect in DNA strand-break repair, and an extraordinary baseline frequency of sister-chromatid exchange. Mutat Res 1982;95:427–440. [PubMed: 6889677]
24. Bol SA, van Steeg H, van Oostrom CT, Tates AD, Vrieling H, de Groot AJ, Mullenders LH, van Zeeland AA, Jansen JG. Nucleotide excision repair modulates the cytotoxic and mutagenic effects of *N*-butyl-*N*-nitrosourea in cultured mammalian cells as well as in mouse splenocytes *in vivo*. Mutagenesis 1999;14:317–322. [PubMed: 10374999]

25. Coryell VH, Stearns DM. Molecular analysis of hprt mutations induced by chromium picolinate in CHO AA8 cells. Mutat Res 2006;610:114–123. [PubMed: 16877033]
26. Coryell VH, Stearns DM. Molecular analysis of hprt mutations generated in Chinese hamster ovary EM9 cells by uranyl acetate, by hydrogen peroxide, and spontaneously. Mol Carcinog 2006;45:60–72. [PubMed: 16299811]
27. Thompson LH, Salazar EP, Brookman KW, Hoy CA. Hypersensitivity to cell killing and mutation induction by chemical carcinogens in an excision repair-deficient mutant of CHO cells. Mutat Res 1983;112:329–344. [PubMed: 6656796]
28. Meschini R, Marotta E, Berni A, Filippi S, Fiore M, Mancinelli P, Natarajan AT, Palitti F. DNA repair deficiency and BPDE-induced chromosomal alterations in CHO cells. Mutat Res 2008;637:93–100. [PubMed: 17720205]
29. Op, hV; Hees-Stuivenberg, S.; van Zeeland, AA.; Jansen, JG. Effect of nucleotide excision repair on hprt gene mutations in rodent cells exposed to DNA ethylating agents. Mutagenesis 1997;12:417–424. [PubMed: 9412994]
30. Cai YN, Wu MH, Ludeman SM, Grdina DJ, Dolan ME. Role of $O^6$-alkylguanine-DNA alkyltransferase in protecting against cyclophosphamide-induced toxicity and mutagenicity. Cancer Res 1999;59:3059–3063. [PubMed: 10397244]
31. Lao Y, Villalta PW, Sturla SJ, Wang M, Hecht SS. Quantitation of pyridyloxobutyl DNA adducts of tobacco-specific nitrosamines in rat tissue DNA by high-performance liquid chromatography-electrospray ionization-tandem mass spectrometry. Chem Res Toxicol 2006;19:674–682. [PubMed: 16696570]
32. Thomson NM, Mijal RS, Ziegel R, Fleischer NL, Pegg AE, Tretyakova N, Peterson LA. Development of a quantitative liquid chromatography/electrospray mass spectrometric assay for a mutagenic tobacco-specific nitrosamine-derived DNA adduct, $O^6$-[4-oxo-4-(3-pyridyl)butyl]-2′-deoxyguanosine. Chem Res Toxicol 2004;17:1600–1606. [PubMed: 15606135]
33. O'Neill JP, Brimer PA, Machanoff R, Hirsch GP, Hsie AW. A quantitative assay of mutation induction at the hypoxanthine-guanine phosphoribosyl transferase locus in Chinese hamster ovary cells (CHO/HGPRT system): development and definition of the system. Mutat Res 1977;45:91–101. [PubMed: 917038]
34. O'Neill JP, Hsie AW. Phenotypic expression time of mutagen-induced 6-thioguanine resistance in Chinese hamster ovary cells (CHO/HGPRT system). Mutat Res 1979;59:109–118. [PubMed: 372789]
35. Sandercock LE, Hahn JN, Li L, Luchman HA, Giesbrecht JL, Peterson LA, Jirik FR. Mgmt deficiency alters the in vivo mutational spectrum of tissues exposed to the tobacco carcinogen 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK). Carcinogenesis 2008;29:866–874. [PubMed: 18281247]
36. Reardon JT, Sancar A. Purification and characterization of Escherichia coli and human nucleotide excision repair enzyme systems. Methods Enzymol 2006;408:189–213. [PubMed: 16793370]
37. Zhao X, Kao JL, Taylor JS. Preparation and characterization of a deoxyoligonucleotide 49-mer containing a site-specific thymidylyl-(3′,5′)-deoxyadenosine photoproduct. Biochemistry 1995;34:1386–1392. [PubMed: 7827086]
38. Wang L, Spratt TE, Pegg AE, Peterson LA. Synthesis of DNA oligonucleotides containing site-specifically incorporated $O^6$-[4-oxo-4-(3-pyridyl)butyl]guanine and their reaction with $O^6$-alkylguanine-DNA alkyltransferase. Chem Res Toxicol 1999;12:127–131. [PubMed: 10027788]
39. Xu Z, Yu Y, Schwartz JL, Meltz ML, Hsie AW. Molecular nature of spontaneous mutations at the hypoxanthine-guanine phosphoribosyltransferase (hprt) locus in Chinese hamster ovary cells. Environ Mol Mutagen 1995;26:127–138. [PubMed: 7556109]
40. Mijal RS, Thomson NM, Fleischer NL, Pauly GT, Moschel RC, Kanugula S, Fang Q, Pegg AE, Peterson LA. The repair of the tobacco-specific nitrosamine derived adduct $O^6$-[4-oxo-4-(3-pyridyl) butyl]guanine by $O^6$-alkylguanine-DNA alkyltransferase variants. Chem Res Toxicol 2004;17:424–434. [PubMed: 15025514]
41. Boyle JM, Saffhill R, Margison GP, Fox M. A comparison of cell survival, mutation and persistence of putative promutagenic lesions in Chinese hamster cells exposed to BNU or MNU. Carcinogenesis 1986;7:1981–1985. [PubMed: 3779894]

42. Hashimoto K, Ohsawa K, Kimura M. Mutations induced by 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) in the lacZ and cII genes of Muta Mouse. Mutat Res 2004;560:119–131. [PubMed: 15157650]
43. Sturla SJ, Scott J, Lao Y, Hecht SS, Villalta PW. Mass spectrometric analysis of relative levels of pyridyloxobutylation adducts formed in the reaction of DNA with a chemically activated form of the tobacco-specific carcinogen 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone. Chem Res Toxicol 2005;18:1048–1055. [PubMed: 15962940]
44. Bhanot OS, Grevatt PC, Donahue JM, Gabrielides CN, Solomon JJ. In vitro DNA replication implicates O2-ethyldeoxythymidine in transversion mutagenesis by ethylating agents. Nucleic Acids Res 1992;20:587–594. [PubMed: 1741292]
45. Fan J, Wilson DM III. Protein-protein interactions and posttranslational modifications in mammalian base excision repair. Free Radic Biol Med 2005;38:1121–1138. [PubMed: 15808410]
46. Airoldi L, Galli A, Magagnotti C, Bagnati R, Lolli M, Fanelli R. Urinary excretion of $O^6$-butylguanine after the administration of *N*-nitroso-*N*-butylurea in rats. Cancer Res 2003;52:6699–6701. [PubMed: 1423317]
47. Simonelli V, Narciso L, Dogliotti E, Fortini P. Base excision repair intermediates are mutagenic in mammalian cells. Nucleic Acids Res 2005;33:4404–4411. [PubMed: 16077026]
48. Bignami M, Dogliotti E, Aquilina G, Zijino A, Wild CP, Montesano R. $O^6$-methyltransferase-deficient and -proficient CHO cells differ in their responses to ethyl- and methyl-nitrosourea-induced DNA alkylation. Carcinogenesis 1989;10:1329–1332. [PubMed: 2736722]
49. Dolan ME, Norbeck L, Clyde C, Hora NK, Erickson LC, Pegg AE. Expression of mammalian $O^6$-alkylguanine-DNA alkyltransferase in a cell line sensitive to alkylating agents. Carcinogenesis 1989;10:1613–1619. [PubMed: 2766456]

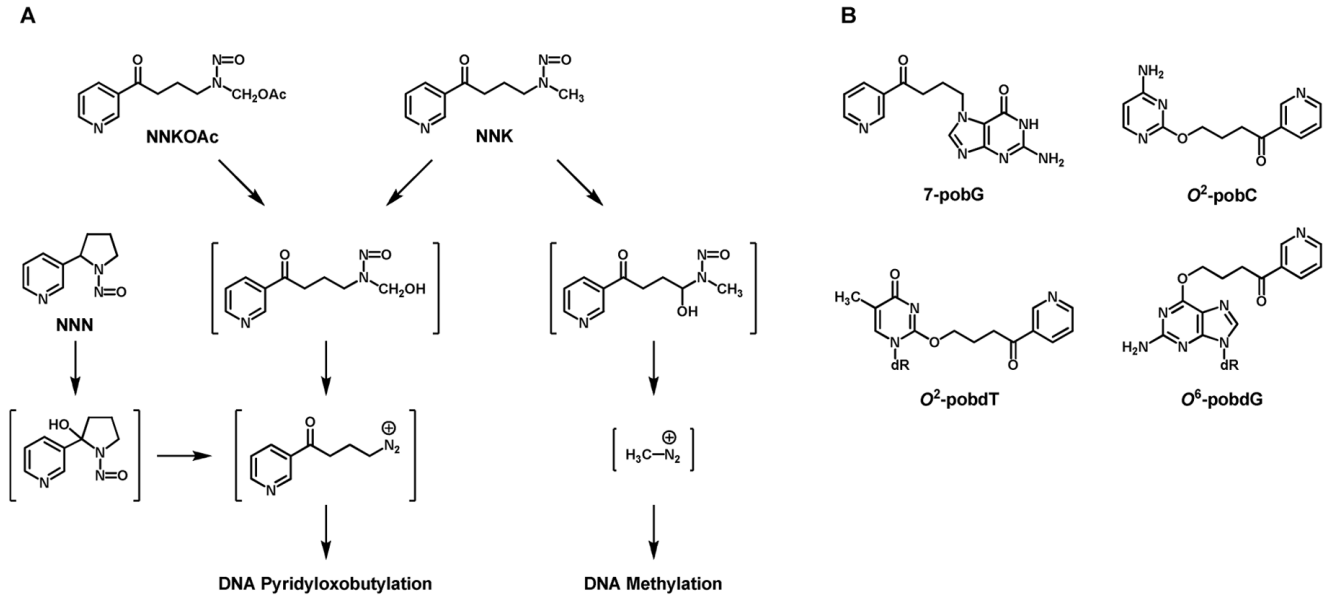


**Figure 1.**
A. Proposed activation pathways for NNN, NNK and NNKOAc. B. Structures of characterized pyridyloxobutyl DNA adducts.

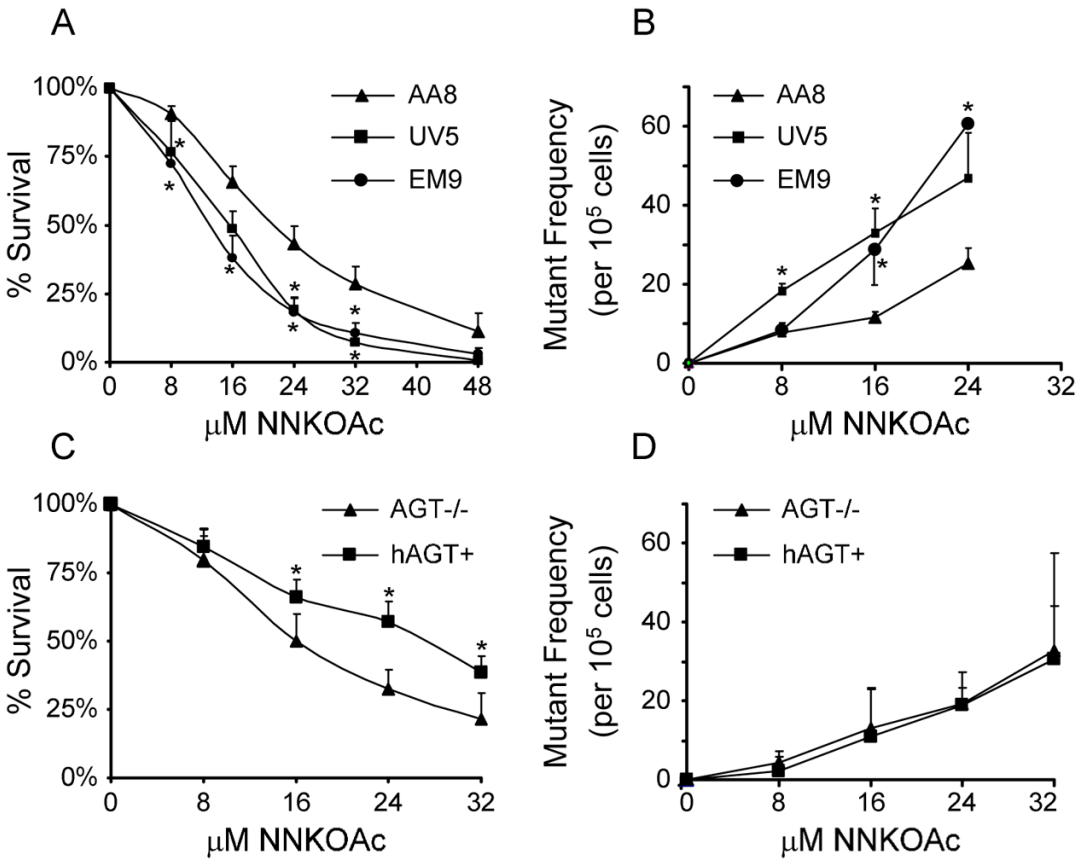


**Figure 2. Cytotoxicity (A, C) and *hprt* mutagenic activity (B, D) in CHO cell lines following a 1 h treatment with increasing concentrations of NNKOAc**

The values represent the average of four separate experiments. The error bars represent the standard deviation. AA8: normal NER, no AGT; UV5: deficient in NER, no AGT; EM9: deficient in XRCC1 (BER), no AGT; $CHO^{pcDNA3}$: empty vector; $CHO^{AGT}$: vector containing hAGT. * indicates that the treated cell lines were statistically significantly different from the parent cell lines at that concentration, $p < 0.05$.

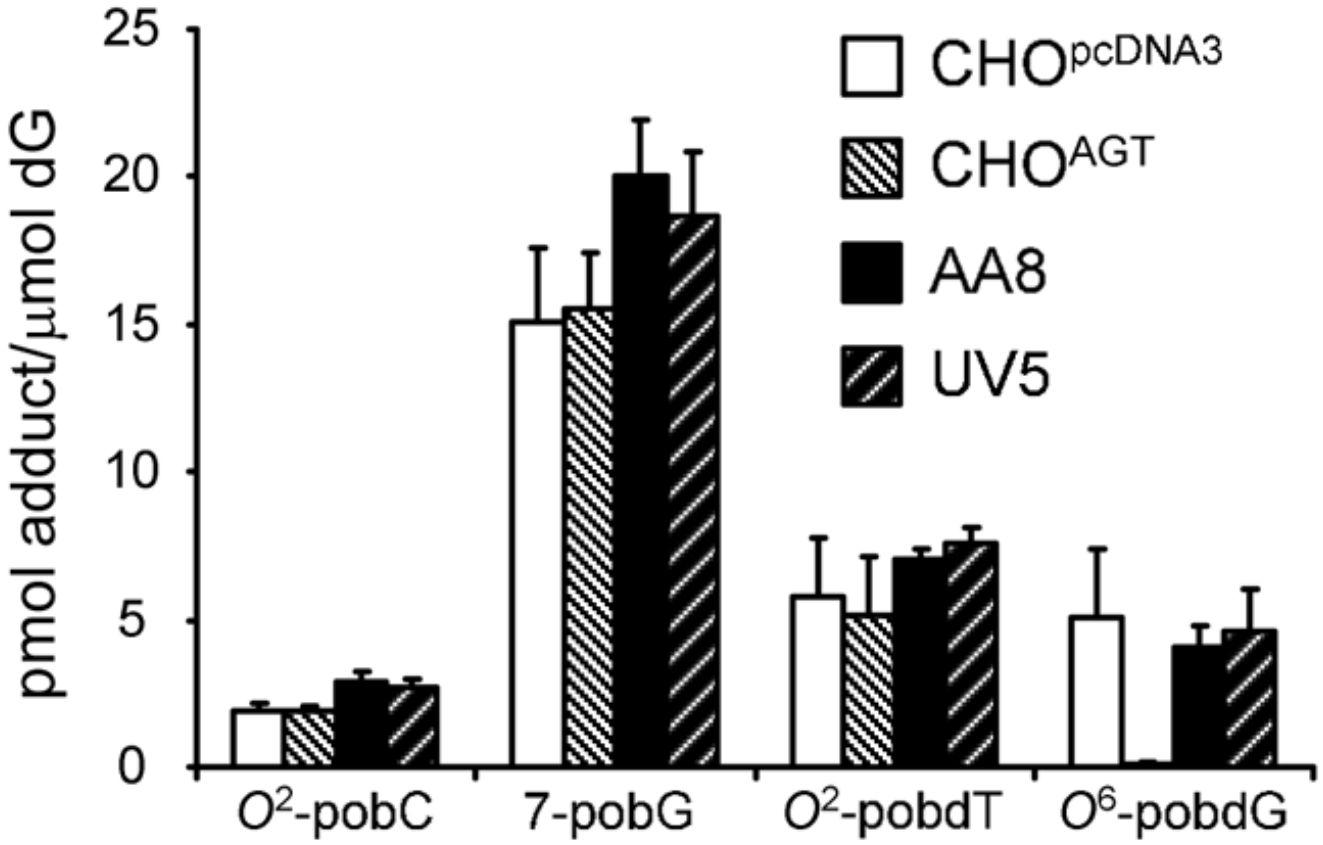


**Figure 3. Initial levels of pyridyloxobutyl DNA adducts in CHO cell lines following a 1 h treatment with 32 μM NNKOAc**

The values represent averages of 3 replicates. The error bars represent the standard deviation. AA8: normal NER, no AGT; UV5: deficient in NER, no AGT; $CHO^{pcDNA3}$: empty vector; $CHO^{AGT}$: vector containing hAGT. Adduct levels were determined by LC-MS/MS analysis of enzymatic hydrolysates of DNA isolated from NNKOAc-treated CHO cell lines.

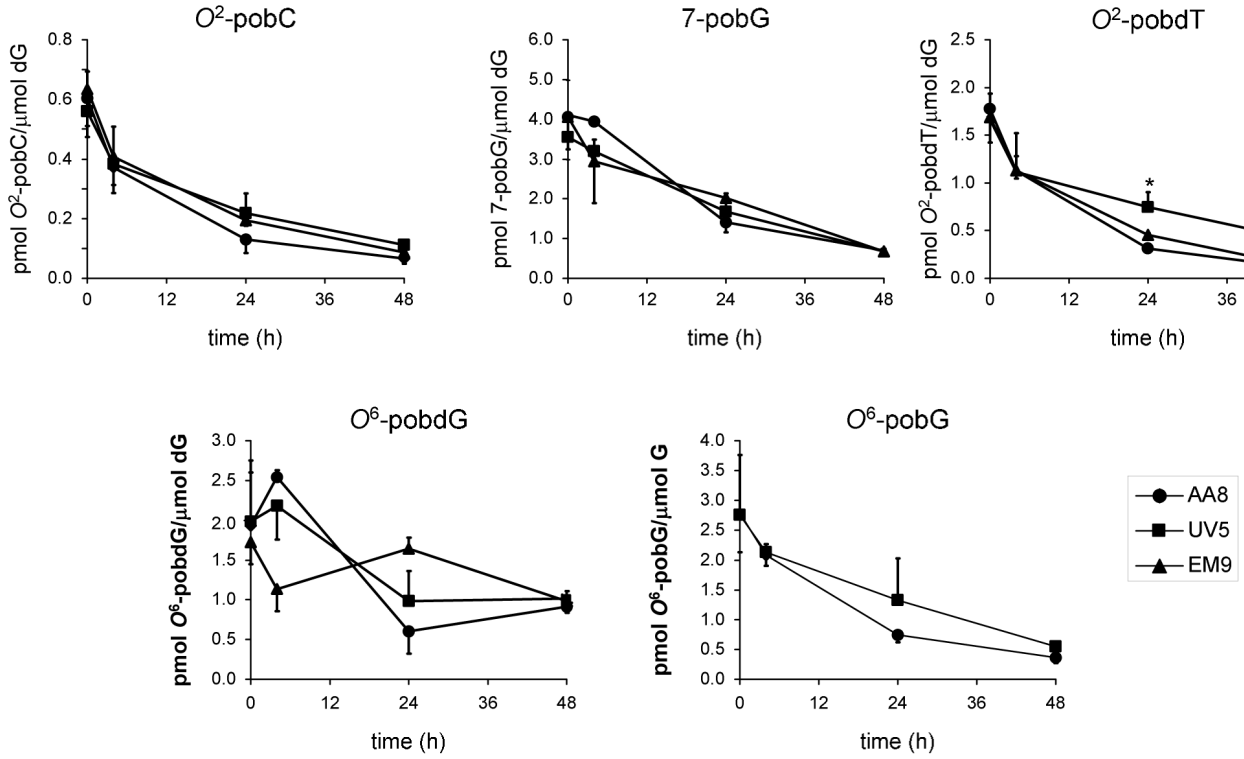


**Figure 4. Time course of adduct repair following a 1 h exposure to 8 μM NNKOAc**

The values equal the average of 3–5 replicates. The error bars represent the standard deviation. Adduct levels are normalized relative to the dG ($O^2$-pobC, 7-pobG, $O^2$-pobdT and $O^6$-pobdG) or G ($O^6$-pobG) concentrations in the enzyme or acid hydrolysates, respectively. AA8: normal NER, no AGT; UV5: deficient in NER, no AGT; EM9: deficient in XRCC1 (BER), no AGT; $CHO^{pcDNA3}$: empty vector; $CHO^{AGT}$: vector containing hAGT. * indicates that the treated cell lines were statistically significantly different from the parent cell lines at that concentration, $p < 0.05$.

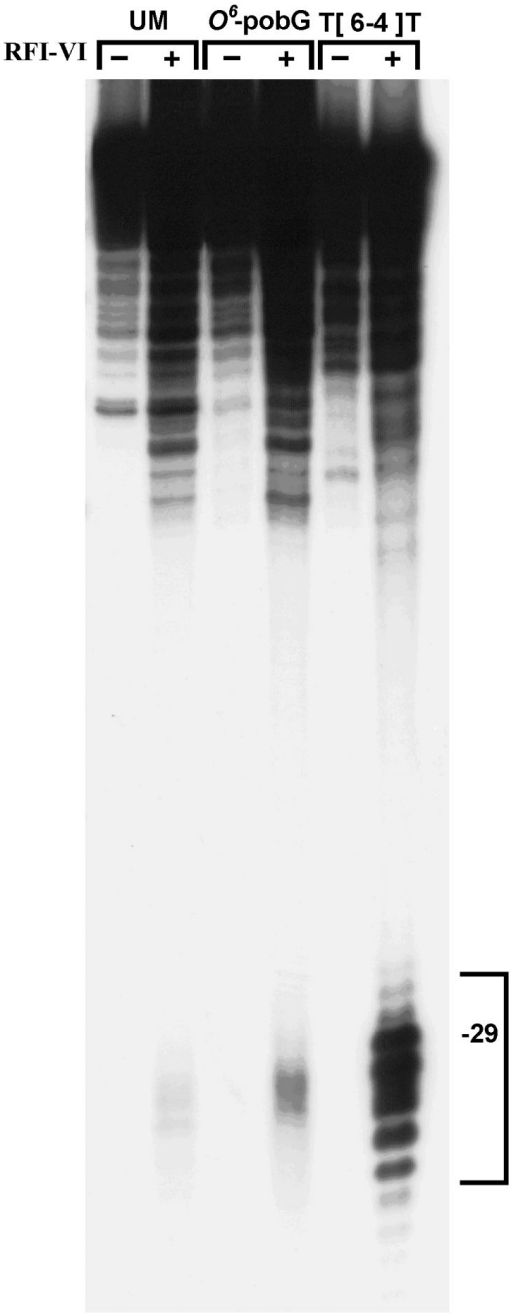


**Figure 5. Excision of $O^6$-pobdG adduct by human repair factors**

Substrate DNA (0.8 nM) was incubated with RPA (140 nM), XPA (65 nM), XPC•hR23B (5 nM), TFIIH (~ 12 nM), XPF•ERCC1 (4 nM), and XPG (3 nM) for 2 h at 30°C. DNA alone (lanes 1, 3, 5) and complete reactions (lanes 2, 4, 6) are shown for undamaged DNA (lanes 1–2), $O^6$-pobdG DNA (lanes 3–4), and T[6-4]T photoproduct (lanes 5–6). Brackets indicate excision products visualized by autoradiography after resolution in 10% sequencing gel. Levels of excision in this experiment were 0.8% (UM), 1.1% ($O^6$-pobdG), and 32.7% T[6-4]T.

**Table 1**

Repair characteristics of Chinese Hamster ovary cell lines

| Cell line | Repair Characteristics |
|---|---|
| AA8 | Lacks AGT |
| UV5 | Lacks AGT and ERCC2 (NER) |
| EM9 | Lacks AGT and XRCC1 (BER) |
| $CHO^{pcDNA3}$ | Lacks AGT |
| $CHO^{AGT}$ | Express human AGT |

**Table 2**

Base substitution mutations observed in 6-thioguanine-resistant mutant colonies

| | Number of Clones (% of base substitution) 24 μM NNKOAc | | | | | Spontaneous | |
|---|---|---|---|---|---|---|---|
| **Class of mutations** | **AA8** | **UV5** | **EM9** | **CHO$^{pcDNA3}$** | **CHO$^{AGT}$** | **AA8[1]** | **EM9[2]** |
| Transitions: | | | | | | | |
| A:T→G:C | 1 (4) | 3 (13) | 3 (13) | 3 (13) | 5 (12) | 2 (2) | 0 (0) |
| G:C→A:T | 6 (25) | 4 (17) | 4 (17) | 4 (17) | 2 (5) | 10 (16) | 2 (16) |
| Transversions: | | | | | | | |
| A:T→C:G | 15 (63) | 8 (35) | 12 (52) | 16 (70) | 32 (74) | 4 (12) | 0 (0) |
| A:T→T:A | 1 (4) | 5 (22) | 4 (17) | 0 | 2 (5) | 2 (6) | 1 (8) |
| G:C→C:G | 0 (0) | 1 (4) | 0 (0) | 0 | 0 | 4 (12) | 1 (8) |
| G:C→T:A | 1 (4) | 2 (9) | 0 (0) | 0 | 2 (5) | 12 (35) | 9 (69) |
| total | 24 | 23 | 23 | 23 | 43 | 34 | 13 |

[1] As previously reported (39).

[2] As previously reported (26)