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Formation and repair of pyridyloxobutyl DNA adducts and their relationship to tumor yield in A/J mice

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Abstract

The nitrosamine 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) is a known human carcinogen. It generates methyl and pyridyloxobutyl DNA adducts. The role of the methyl DNA adducts has been well-established in the tumorigenic properties of NNK. However, the role of the pyridyloxobutyl DNA adducts is unclear. Four pyridyloxobutyl DNA adducts have been characterized: 7-[4-3-(pyridyl)-4-oxobut-1-yl]guanine (7-pobG), O^2-[4-3-(pyridyl)-4-oxobut-1-yl]-cytodine (O^2-pobC), O^2-[4-3-(pyridyl)-4-oxobut-1yl]thymidine (O^2-pobdT), and O^6-[4-3-(pyridyl)-4-oxobut-1-yl]-2'-deoxyguanosine (O^6-pobdG). Mutagenic O^6-pobdG is thought to contribute to the tumorigenic properties of the pyridyloxobutylation pathway. It is repaired by O^6-alkylguanine-DNA alkyltransferase (AGT). To explore the role of O^6-pobdG formation and repair in the tumorigenic properties of NNK, A/J mice were given single or multiple doses of the model pyridyloxobutylating agent 4-(acetoxymethyl-nitrosamino)-1-(3-pyridyl)-1-butanone (NNKOAc) in the presence or absence of the AGT depletor, O^6-benzylguanine. Levels of the four pyridyloxobutyl DNA adducts were measured in the lung at 8, 48 or 96 h following treatment and compared to the lung tumorigenic activity of these treatments. AGT depletion had only a modest effect on the levels of O^6-pobdG and did not increase tumor formation. Three pyridyloxobutyl DNA adducts, 7-pobG, O^2-pobdT, and O^6-pobdG, persisted in lung DNA at significant levels for up to 96 h post-treatment, suggesting that all three adducts may contribute to the tumorigenic properties of NNK.

Keywords

AGT; DNA adduct; lung tumors; tobacco-specific nitrosamines; NNK

Introduction

The tobacco-specific nitrosamine 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) is a potent lung specific carcinogen in rodents[1] and is listed as a human carcinogen (Group 1) by the International Agency for Cancer Research.[2] NNK carcinogenesis requires cytochrome P450-catalyzed oxidation to DNA reactive metabolites.[3] There are two α-hydroxylation pathways that lead to DNA adducts (Figure 1). Methylene hydroxylation creates methanediazohydroxide which alkylates DNA to form 7-methylguanine (7-mG), O^6-methylguanine (O^6-mG), and O^4-methylthymidine (O^4-mT).[1] Methyl hydroxylation produces 4-oxo-4-(3-pyridyl)-1-butanediazohydroxide which generates 7-[4-3-(pyridyl)-4-oxobut-1-yl]guanine (7-pobG), O^2-[4-3-(pyridyl)-4-oxobut-1-yl]-cytodine (O^2-pobC), O^2-

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[4-3-(pyridyl)-4-oxobut-1yl]thymidine (O^2-pobdT), and O^6-[4-3-(pyridyl)-4-oxobut-1-yl]-2'-deoxyguanosine (O^6-pobdG) as well as adducts that release 4-hydroxy-1-(3-pyridyl)-1-butanone (HPB).[4–6] Both of these pathways influence the carcinogenic nature of NNK.[7–9]

While the contribution of O^6-mG to the carcinogenic properties of NNK is well-established,[1] the role of specific adducts in the carcinogenic properties of the pyridyloxobutylation pathway has not been fully investigated. Experimental evidence suggests that pyridyloxobutyl DNA adducts may contribute to the carcinogenic properties of NNK. First, pyridyloxobutyl DNA adducts have been detected in DNA from tissues of NNK-treated rats[10–12] and mice.[13] Levels of HPB-releasing DNA adducts in type II cells are correlated to lung tumor formation in rats.[9] Second, the model pyridyloxobutylating agent 4-(acetoxymethylnitrosamino)-1-(3-pyridyl)-1-butanone (NNKOAc) caused lung tumors in A/J mice in either single or chronic dosing regimens.[8,14] Esterase catalyzed hydrolysis of NNKOAc generates the same pyridyloxobutylating intermediate formed following methyl hydroxylation of NNK (Figure 1), and therefore allows for the exclusive exploration of this pathway in the absence of the methylating pathway.[8,15,16] Third, pyridyloxobutylating agents are mutagenic in the Ames assay[17,18] and in the *Hprt* gene of Chinese hamster ovary (CHO) cells.[19] Finally, pyridyloxobutyl DNA adducts accumulate in normal lung tissue of smokers with lung cancer but not in lung tissue from smokers without lung cancer.[20]

One adduct that is likely to contribute to the mutagenic properties of the pyridyloxobutylation pathway is O^6-pobdG. Site-specific mutagenesis studies demonstrated that it produced GC to AT transitional mutations in bacteria.[21] In human cells, a small number of GC to TA transversions and more complex mutations were also observed.[21] Consistent with the potential importance of O^6-pobdG to the tumorigenic activity of NNKOAc, GC to AT transitions and GC to TA transversions were observed in the K-*ras* oncogene of tumors induced by NNKOAc in A/J mice.[14]

The repair of pyridyloxobutyl DNA adducts is critical to preventing their mutagenic properties. Removal of O^6-pobdG from DNA is facilitated by O^6-alkylguanine DNA alkyltransferase (AGT) in a reaction that involves transfer of the alkyl group from the O^6-position of guanine to a cysteinyl residue on the protein.[4,13,19,22] Importantly, AGT protects cells from the mutagenic properties of O^6-pobdG.[19,21,23]

In this study, we tested the hypothesis that pulmonary levels of O^6-pobdG are correlated to lung tumor formation in NNKOAc-treated A/J mice. The A/J mouse lung tumor model is a useful tool for the investigation of initiation mechanisms involved in nitrosamine carcinogenesis.[8,13,16,24,25] It is particularly valuable when using activated forms of nitrosamines, such as NNKOAc, since these animals will get lung tumors more rapidly than tumors at the injection site.[14] Previously, we employed this animal model to link O^6-mG formation and repair with lung tumor formation in mice treated with DNA methylating nitrosamines.[8,16] Therefore, if O^6-pobdG is critical for the formation of lung tumors, we proposed that there would be a correlation between levels of O^6-pobdG and the number of lung adenomas in animals treated with the model pyridyloxobutylating agent NNKOAc. Since AGT repairs O^6-pobdG, we also probed the role of AGT in adduct repair and tumor formation in the A/J mouse by co-administration of an AGT depletor, O^6-benzylguanine (O^6-bzG).[26] O^6-bzG treatment resulted in increased levels of O^6-pobdG in both lung and liver DNA from NNKOAc-treated mice.[13] Therefore, we proposed that AGT depletion may enhance the tumorigenic effects of NNKOAc.

Two dosing regimens were employed in this study. In the single dosing regimen, one dose of 4.2 or 10 μmol NNKOAc was administered in the presence or absence of 2.5 μmol O^6-bzG.

Single doses of 4.2 or 10 μmol NNKOAc led to a small but significant increase in lung tumor formation sixteen weeks following exposure in previous studies.[8] A dose of 4.2 μmol NNKOAc generated pulmonary levels of HPB-releasing DNA adducts comparable to those caused by a 10 μmol NNK 24 h after treatment.[8] Earlier studies demonstrated that a dose of 2.5 μmol O^6-bzG depleted AGT in lungs and increased the levels of O^6-pobG in lung DNA from NNKOAc-treated mice.[13,16] This amount of O^6-bzG increased the levels of O^6-mG as well as lung tumor yields in this animal model.[16] In the chronic treatment group, mice were given either 1.5 or 2.0 μmol NNKOAc in the presence or absence of 2.5 μmol O^6-bzG. In a previous study, chronic treatment of 2.5 μmol NNKOAc three times weekly for a total of 17 injections triggered the formation of an average of 13 tumors/mouse 35 weeks later.[14] Lower doses (1.5 and 2.0 μmol) were used in the current study since multiple doses of 2.5 μmol NNKOAc were toxic to mice.[14] A single 1.5 μmol dose of NNKOAc is predicted to yield the same overall lung DNA adducts as a 10 μmol dose of NNK.[8] Adduct levels were also measured following a single 10 μmol dose of NNK for comparison purposes; this dose of NNK induced 8–10 lung tumors/mouse in past studies.[1,8,16]

Materials and Methods

Caution

NNK and NNKOAc are carcinogenic. They should be handled with extreme caution in a well-ventilated hood and with personal protective equipment.

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, [pyridine-$^2H_4$]O^2-[4-(3-pyridyl)-4-oxobut-1-yl]-2'-deoxythymidine, and [1,2,2-$^2H_3$]O^6-[4-(3-pyridyl)-4-oxobut-1-yl]-2'-deoxyguanosine were synthesized as previously described.[10,27,28] NNK was purchased from Toronto Research Chemicals Inc (North York, Ontario). Micrococcal nuclease and phosphodiesterase II were obtained from Worthington Biochemical Corporation (Lakeswood, NJ). Alkaline phosphatase was purchased from Roche Molecular Biochemicals (Indianapolis, IN). DNA isolation kits were purchased from Qiagen (Valencia, CA). T4 polynucleotide kinase was purchased from Affymetrix (Santa Clara, CA). [γ-^{33}P]ATP was from Perkin Elmer Life and Analytical Sciences (Waltham, MA). Modified oligonucleotide substrates containing O^6-methylguanine were purchased from Operon Biotechnologies Inc. (Huntsville, AL) and their unmodified compliments were purchased from the University of Minnesota MicroChemical Facility (Minneapolis, MN). All other chemicals and solvents were acquired from Sigma-Aldrich Chemical Co (Milwaukee, WI) or Fisher Scientific (Fairlawn, NJ).

Animals—The study was approved by the University of Minnesota Institutional Animal Care and Use Committee and performed in accordance with NIH guidelines. Female A/J mice, 5–7 weeks old, were purchased from Jackson Laboratories (Bar Harbor, ME) and housed five animals per cage under standard conditions and maintained on American Institute of Nutrition-76-A-modified diet (Dyets, Inc., Bethlehem, PA). They were randomly divided into treatment groups and acclimated to the facility for one week prior to carcinogen treatment. Body weights were recorded throughout the study. No differences were observed between treatment and control groups (data not shown).

Single Dose NNKOAc Treatment: Groups of mice were given a single i.p. injection of 4.2 or 10 μmol NNKOAc in saline (0.2 mL). Mice also received 4 daily injections of either phosphate buffered saline (PBS) containing 40% polyethylene glycol 400 (PEG-400) (0.2 mL) or 2.5 μmol O^6-bzG in PBS containing 40% PEG-400 (0.2 mL). The first injection occurred immediately prior to the nitrosamine injection. Control mice were given a single

i.p. injection of saline (0.2 mL) as well as four daily i.p. injections of PBS containing 40% PEG-400 (0.2 mL) or 2.5 μmol O^6-bzG in PBS containing 40% PEG-400 (0.2 mL). In the DNA adduct studies, groups of five mice were sacrificed 8, 48, or 96 h post-nitrosamine injection. Lungs were removed and immediately frozen on dry ice and stored at −80°C. In the tumor bioassay, groups of 20 mice were sacrificed 16 weeks following the nitrosamine injection. Lungs were removed and fixed in 10% formalin in phosphate buffer. The lobes were separated and surface nodules were counted under a microscope.

Single Dose NNK Treatment: Groups of 5 mice were given a single i.p. injection of 10 μmol NNK in saline (0.2 mL). Mice were sacrificed 8, 48, or 96 h post-injection. Lungs were removed and immediately frozen on dry ice and stored at −80 °C.

Chronic Dose NNKOAc Treatment: Groups of mice were given an i.p. injection of 1.5 or 2.0 μmol NNKOAc in saline (0.2 mL) three times week for 7 weeks. Mice also received an i.p. injection of PBS containing 40% PEG-400 (0.2 mL) or 2.5 μmol O^6-bzG in PBS containing 40% PEG-400 (0.2 mL) immediately prior to the nitrosamine. Control mice were given an i.p. injection of PBS containing 40% PEG-400 (0.2 mL) or 2.5 μmol O^6-bzG in PBS containing 40% PEG-400 (0.2 mL) followed by an i.p. injection of saline (0.2 mL) three times a week for 7 weeks. For DNA adduct analysis, groups of 5 mice in the controls and 1.5 μmol NNKOAc group were sacrificed 8 and 48 h following the 3rd and 12th injections of NNKOAc. In all treatment groups, groups of 5 mice were sacrificed 8 or 48 h following the 21st injection. Lungs were removed and immediately frozen on dry ice and stored at −80 °C. In the tumor bioassay, groups of 20 mice were sacrificed 35 weeks following the first injection. Lungs were removed and fixed in 10% formalin in phosphate buffer. The lobes were separated and surface nodules were counted under a microscope.

Quantitation of DNA adducts by HPLC-ESI-MS/MS—DNA was isolated from half of each mouse lung following the Qiagen Midi Protocol (Qiagen, Valencia, CA). DNA was subjected to enzymatic hydrolysis according to published methods.[10] The hydrolysates were analyzed for DNA adducts by HPLC-ESI-MS/MS. The hydrolysates were separated on a 300 μm × 100 mm BEH 130 C18 column at 50 °C with a nanoACQUITY UltraPerformance LC System (Waters Corporation, Milford, MA) into a Thermo Electron Quantum Ultra AM triple quadrupole mass spectrometer (Thermo Election, San Jose, CA) in positive ion mode. The column was eluted with a linear gradient from 10% acetonitrile to 50% acetonitrile in 15 mM ammonium acetate over a period of 11 min, then 50% acetonitrile for 2 min, returning to 10% acetonitrile in 2 min (flow rate: 10 μL/min). Pyridyloxobutyl DNA adducts and deuterated internal standards were detected by monitoring the formation of the pyridyloxobutyl ion from the parent ion by selected reaction monitoring (SRM): O^2-pobC at *m/z* 259.1→148.1 and [pyridine-$^2H_4$]O^2-pobC at *m/z* 263.1→152.1, 7-pobG at *m/z* 299.1→148.1 and [pyridine-$^2H_4$]7-pobG at *m/z* 303.1→152.1, O^2-pobdT at *m/z* 390.1→148.1 and [pyridine-$^2H_4$]O^2-pobdT at *m/z* 394.1→152.1, O^6-pobdG at *m/z* 415.1→148.1 and [1,2,2-$^2H_3$]O^6-pobdG at *m/z* 418.1→151.1. Levels of adduct were expressed relative to the 2'-deoxyguanosine concentration in the hydrolysates, which were quantitated by high-pressure liquid chromatography with UV detection.[28]

AGT Assay: Tissue extracts were prepared as previously reported.[29] Briefly, one-half of an A/J mouse lung was homogenized in ice cold cell extract buffer (78 mM HEPES, 1.1 mM EDTA, 1 mM DTT, and 5% glycerol, pH 7.8; total volume 250 μL) at 4 °C in a tissue grinder. The homogenate was sonicated 6 × 2 s at 4 °C. The homogenates were centrifuged 2 min (16000 × *g*) at 4 °C. The resulting supernatants were assayed immediately for AGT activity. Protein content was measured by the Bradford method with the Bio-Rad Protein Assay Reagent (Bio-Rad, Hercules, CA).

AGT activity was measured using a modification of a previously described protocol.[30,31] First, an O^6-mG modified oligonucleotide (AACAGCCATATO^6-mGGCCC) was [^{33}P]-endlabeled with [^{33}P]-ATP by T4 polynucleotide kinase in T4 polynucleotide kinase reaction buffer for 30 min at 37 °C. T4 polynucleotide kinase was inactivated by heating for 10 min at 65 °C. The oligonucleotide was purified on a G25 spin column (GE Healthcare, Waukesha, WI). It was then annealed with 2-fold excess of the compliment strand by heating from 65 °C to 80 °C, then holding at 80 °C for 2 min before slowly cooling to room temperature over several hours.

Reactions with tissue extracts were initiated by the addition of lung homogenates to a 100 μL solution of [^{33}P]-endlabeled oligonucleotide duplex (194 fmol) in 50 mM Tris, pH 7.8, containing 1 mM EDTA and 1 mM DTT. After 30 min at 37 °C, the protein was removed by serial extraction with 25:24:1 phenol:chloroform:isoamyl alcohol and 24:1 chloroform:isoamyl alcohol using MaXtract High Density tubes (Qiagen,Germantown, MD) according to manufacturer instructions. Following extraction, the samples were adjusted to 0.1 M NaOH. If not analyzed immediately, samples were stored at −80 °C until use. The reaction mixture was analyzed by HPLC with radioflow detection using a 4 × 250 mm DNAPac PA100 (Dionex) anion-exchange column that was eluted with a NaCl gradient in 10 mM NaOH and 2% acetonitrile.[30,31] The extent of repair was expressed as the ratio of the modified peak area over the sum of the unmodified and modified peak areas. Activity was calculated by multiplying the repaired fraction by the total amount of oligonucleotide (194 fmol) and expressed relative to protein concentration.

Statistical Analyses—All statistical analyses were conducted in SAS (Statistical Analysis Software) version 9.2 at the Biostatistics Core of the University of Minnesota Masonic Cancer Center. Pyridyloxobutyl DNA adducts and tumors/mouse were summarized by mean and standard deviation. Pyridyloxobutyl DNA adducts were approximately normally distributed. Analysis of variance (ANOVA) was used to investigate the overall effect of time, the presence of O^6-bzG, and NNKOAc concentration on pyridyloxobutyl DNA adduct levels.[32] ANOVA was also used to investigate the effect of NNKOAc and O^6-bzG on tumors/mouse. Multiple comparisons were adjusted by Bonferroni method. Pearson correlation coefficients were calculated for each of the pyridyloxobutyl DNA adducts with tumors/mouse.[33] The non-parametric Wilcoxon test was used to analyze the AGT activity changes with treatment. The significance level was set to 5%.

Results

Single Dose Adduct Study

To determine the extent of repair, animals were sacrificed 8, 48, and 96 h following a single dose of NNK or NNKOAc alone or in combination with O^6-bzG. In a preliminary study, A/J mice were given a single dose of 4.2 μmol NNKOAc in the presence or absence of a single dose of 2.5 μmol O^6-bzG. At 8 h, co-administration of O^6-bzG elevated the levels of O^6-pobdG roughly two fold with no effect on the levels of the other adducts (data not shown). However, this effect was not sustained since there was no difference in the levels of O^6-pobdG between the two groups at 48 h. Therefore, subsequent experiments were performed with four daily doses of O^6-bzG to ensure consistent AGT depletion.

All four pyridyloxobutyl DNA adducts were detected in lung DNA from mice treated with NNK or NNKOAc, with the highest levels detected at 8 h (Table 1). Pyridyloxobutyl DNA adduct levels were lower in NNK-treated animals than NNKOAc-treated animals (Figure 2, Table 1). However, the relative amounts of each adduct 8 h following treatment with NNKOAc mimicked those seen in mice treated with a tumorigenic dose of NNK: 7-pobG > O^2-pobdT > O^6-pobdG > O^2-pobC (Figure 2).

The only adduct in the 4.2 μmol NNKOAc-treated mice that underwent significant reduction over 96 h was 7-pobG ($P = 0.02$); the decrease in O^6-pobdG levels were not quite significant ($P = 0.07$) (Table 1, Figure 3A and B). The levels of O^2-pobdT were only nominally reduced by 96 h. Multiple injections of O^6-bzG did not significantly increase the levels of O^6-pobdG. As expected, the levels of the other adducts were also not significantly affected by treatment with O^6-bzG since these adducts are not substrates for AGT.

In animals treated with 10 μmol NNKOAc, adduct levels were higher than those observed in mice receiving 4.2 μmol NNKOAc (Table 1, Figure 2). At this higher dose, O^6-pobdG levels were significantly elevated in animals co-administered O^6-bzG at 48 h but not 8 and 96 h (Table 1, Figure 3C and D). At this dose, there was a significant reduction in the levels of all adducts (7-pobG and O^2-pobC, $P < 0.001$; O^6-pobdG, $P = 0.02$ and O^2-pobdT, $P = 0.04$) over time (Figure 3C), with O^2-pobdT being the most persistent.

Adduct removal in the 10 μmol NNK-treated animals was slow with only O^2-pobC undergoing significant reduction over 96 h ($P = 0.01$) (Table 1, Figure 3E).

Chronic Dose Adduct Study

Lung DNA adduct levels were determined 8 and 48 h following 21 injections of 1.5 or 2.0 μmol NNKOAc. Adduct levels were not measured at 96 h because there were not significant differences in O^6-pobdG levels between 48 and 96 h at the lower dose in the single dose study (Figure 3A). A dose response in adduct levels was not observed in this dosing regimen at 8 h. The relative adduct distribution was similar to the single dose 8 h after the last injection (7-pobG > O^2-pobdT > O^6-pobdG > O^2-pobC; Figure 2). The adduct levels at 48 h have not changed that much from those observed at 8 h post-treatment.

Co-administration of O^6-bzG significantly affected O^6-pobdG levels (Table 1, Figure 4). Two-way ANOVA analysis indicated that the presence of O^6-bzG significantly modified the amount of O^6-pobdG at both doses independent of time ($P < 0.01$). Pairwise analysis with Tukey adjustment indicates that the data at 8 h is driving the difference between these two treatment groups at both doses of NNKOAc, since the levels of O^6-pobdG at 48 h post-treatment were independent of the presence of O^6-bzG.

To determine how adduct levels changed over the course of the 7 week treatment regimen, adduct levels were also measured 8 and 48 h following the 3rd and 12th injections of 1.5 μmol NNKOAc in the presence or absence of O^6-bzG. The maximal levels at 8 h were observed at 1 week for 7-pobG and O^2-pobC, 4 weeks for O^6-pobdG, and 7 weeks for O^2-pobdT (Table 1, Figure 5). The levels of O^2-pobC declined after 1 week ($P = 0.03$) whereas 7-pobG levels remained constant. Chronic dosing led to increased levels of O^6-pobdG and O^2-pobdT ($P < 0.01$) with the largest effect observed with O^2-pobdT.

AGT Activity

To confirm that AGT was depleted in A/J mouse lung by co-administration of O^6-bzG, AGT activity was measured in mouse lungs (Figure 6). As expected, a single dose of 2.5 μmol O^6-bzG alone reduced AGT activity almost completely 8 h post-injection.[16] These reduced levels were maintained at 48 and 96 h with daily doses of O^6-bzG. A single dose of 4.2 μmol or 10 μmol NNKOAc alone resulted in a slight reduction in lung AGT, recovering almost to control activity by 96 h. The decrease in AGT activity by 4.2 μmol NNKOAc was similar to those previously reported.[16] Co-administration of O^6-bzG with NNKOAc resulted in enhanced depletion of AGT with complete inactivation at 8 h. In the animals receiving just a single dose of NNKOAc with daily doses of O^6-bzG, the activity recovers slightly by 96 h indicating there was some residual AGT activity in these animals despite multiple treatments of O^6-bzG.

In the chronic treatment regimen, AGT activity was marginally affected by the multiple doses of 1.5 or 2.0 μmol NNKOAc. In both chronic treatment groups, co-administering O^6-bzG with NNKOAc led to no detectable AGT activity at 8 h, recovering to 43–49% of control levels by 48 h.

Animals receiving a single dose of 10 μmol NNK experienced a large reduction in AGT activity at 8 h, with partial recovery by 96 h. This trend is similar to a previous study, which also reported a dramatic sustained reduction in AGT activity following treatment with 10 μmol NNK.[16] The depletion of AGT results from the repair of the AGT substrate adducts, O^6-mG and O^6-pobdG.

Bioassay for Tumorigenicity

The effect of AGT manipulation by O^6-bzG on tumor yield was determined with both the single injection and chronic dosing regimen. As previously reported,[8,14,16] 4.2 and 10 μmol NNKOAc were only weakly tumorigenic in the A/J mouse lung and the tumorigenic activity of this model pyridyloxobutylating agent was enhanced upon multiple doses (Figure 7, Table 2). Four daily doses of O^6-bzG appeared to increase tumor formation in animals receiving 4.2 μmol but not 10 μmol NNKOAc (Figure 7, Table 2); however, this increase was not statistically significant. A similar trend was observed in the chronic dose tumor study where co-administration of O^6-bzG caused a small but not significant increase in tumors/mouse in the mice receiving doses of 1.5 μmol NNKOAc but not 2.0 μmol NNKOAc (Figure 7, Table 2).

Comparison of Pyridyloxobutyl DNA Adduct Levels to Tumorigenicity

There were insufficient data points to get good correlative data between pyridyloxobutyl DNA adduct levels and lung tumor formation. However, there was roughly a linear relationship between each of the four adducts and tumor formation at the longer time points. Linear relationships were observed between the levels of 7-pobG, O^2-pobT and O^6-pobdG at 48 and 96 h post-injection ($R^2 \geq 0.90$, $P \leq 0.01$). In the chronic dosing study, 7-pobG, O^2-pobT and O^6-pobdG were the best correlated to tumors/mouse at 48 h ($R^2 \geq 0.94$, $P \leq 0.01$).

Discussion

Our study was designed to determine if the formation and persistence of O^6-pobdG are correlated to lung tumor formation in NNKOAc-treated A/J mice. Manipulation of AGT activity did not dramatically enhance the levels of O^6-pobdG; a maximum of approximately two fold increase in the levels of O^6-pobdG was observed. However, this increase did not persist in most cases. Therefore, it is not surprising that O^6-bzG did not increase the tumorigenic activity of NNKOAc in either dosing regimen.

Measurement of AGT activity in lungs indicated that the 2.5 μmol dose of O^6-bzG depleted AGT almost completely (Figure 6). However, there was residual activity in the lungs of NNKOAc-treated mice; it is possible that these low levels of AGT were capable of reducing O^6-pobdG over time. Comparison of the relative levels of 7-pobG and O^6-pobdG observed in NNK or NNKOAc-treated mice to that measured in calf thymus DNA treated with NNKOAc *in vitro* confirms that O^6-pobdG is likely being repaired *in vivo* (Table 3). With the exception of the animals receiving 4.2 μmol NNKOAc, this ratio is more than doubled relative to that observed in NNKOAc-treated calf thymus DNA; these ratios might even be higher since 7-pobG is chemically unstable.[5] AGT is probably not the only pathway involved in the repair of O^6-pobdG since it did not accumulate like O^6-mG in AGT knockout mice treated with multiple doses of NNK.[35] It is a poor substrate for nucleotide excision repair (NER).[19] It is not known if this adduct is a substrate for base excision

glycosylases. The possible involvement of other repair pathways for this adduct partially explains why O^6-bzG was not effective in modulating O^6-pobdG repair in NNKOAc-treated mice.

These results do not eliminate a role of O^6-pobdG in the tumorigenic activity of pyridyloxobutylating agents. This adduct persists at significant levels for at least 96 h post-treatment and may contribute to the NNKOAc-induced GC to AT transitions and GC to TA transversions in the 12th codon of K-*ras* in lung tumors from A/J mice.[14] Consistent with this proposal, site-specific mutagenesis studies demonstrated that O^6-pobdG is a potent mutagenic adduct, triggering GC to AT transition and GC to TA transversion mutations in human cells.[21] Furthermore, the mutagenic activity of NNKOAc in *Salmonella typhimurium* YG7108 was correlated to the levels of O^6-pobdG; this assay measures mutation frequency specifically at GC base pairs.[23] Finally, NNKOAc-induced GC to AT transition mutations in the *Hprt* gene in CHO cells were linked to the formation and repair of O^6-pobdG in these cells.[19] Therefore, it is likely that this adduct contributes to mutagenesis *in vivo*.

Pyridyloxobutyl DNA adducts, 7-pobG and O^2-pobdT, are also persistent with the latter adduct being the most stable. These modified bases are present at higher levels than O^6-pobdG at all time points. Therefore, it is plausible that they could also contribute to the overall tumorigenic properties of pyridyloxobutylating agents. Recent studies indicate that O^2-pobdT is a mutagenic adduct. It was associated with TA to AT mutations in CHO cells.[19] More recently, site-specifically incorporated O^2-pobdT produces TA to GC and TA to AT transversion mutations in a bacterial model system.[36] Therefore, this adduct is likely to be important when the activation of oncogenes requires mutations at AT base pairs.

The mutagenic properties of 7-pobG have not been investigated. It is possible that it may contribute to the GC to TA transversions observed in the activated K-*ras* oncogene in lung tumors from NNKOAc-treated A/J mice.[14] 7-Alkylguanine adducts can undergo imidazole ring opening to mutagenic 2,6-diamino-4-hydroxy-5*N*-alkyl-formamidopyrimidine (fapy) adducts; fapy7-methylguanine and aflatoxin B1-fapy adducts caused GC to TA transversion mutations.[37–39] The ability of 7-pobG to form a fapy adduct has not been investigated. But it is worth noting that pyridyloxobutylated DNA contains fapy glycosylase-sensitive adducts.[40] Another possibility is that 7-pobG spontaneously depurinates from DNA, leaving behind abasic sites.[5,41] Since abasic sites within GC base pairs results in GC to AT transition and GC to TA transversion mutations,[42] the possible involvement of 7-pobG in the genotoxic properties of the pyridyloxobutylating pathway should be explored.

It is unlikely that O^2-pobC contributes significantly to the overall tumorigenic activity of the pyridyloxobutylation pathway. It spontaneously depyrimidates from DNA, resulting in abasic sites just like 7-pobG. However, the levels of this adduct are low and it is removed from DNA. A comparison of the levels of 7-pobG to this adduct indicate that the relative levels of this adduct are, in general, 10 times lower than what is reported in *in vitro* alkylated DNA (Table 3).

Our studies confirm that the DNA pyridyloxobutylation pathway is weakly carcinogenic in the A/J mouse lung when limited to a single exposure. One possible explanation for this weak activity is the relatively low levels of lung DNA pyridyloxobutyl DNA adducts formed. Smaller doses of a model methylating agent, acetoxymethylmethylnitrosamine, generated much higher levels of methyl DNA damage than NNKOAc-derived pyridyloxobutyl DNA adducts reported in this current study.[8,16] These comparisons suggest that the efficiency of DNA alkylation may be reduced for the pyridyloxobutylating diazohydroxide intermediate relative to the methylating analog. This is similar to what has been observed in *in vitro* DNA alkylation reactions.[46]

NNKOAc was significantly more carcinogenic when multiple doses are administered. The observed differences in accumulation of the specific adducts is likely influenced by the rates of removal of each adduct from DNA with O^2-pobdT being the most persistent of the four adducts. While NER appears to be is an important repair pathway for this adduct in mammalian cells,[19] A/J mouse lung has low NER activity.[47] In addition, NNK exposure reduced lung NER activity by 30–45% 4 and 24 h following treatment.[47] It is not known whether NNKOAc has a similar effect on NER in A/J mouse lungs. The loss of 7-pobG and O^2-pobC from lung DNA can be partially explained by the ability of these adducts to spontaneously depurinate or depyrimidate from DNA, leaving behind abasic sites.[5,41] It is also possible that they are actively repaired. *Bacillus cereus* 3-methyladenine DNA glycosylase has been shown to excise both 7-pobG and O^2-pobC whereas human alkyladenine glycosylase did not.[48] Pyridyloxobutylated DNA contains fapy glycosylase-sensitive adducts and it was proposed that 7-pobG was a possible substrate.[40] However, the ability of specific pyridyloxobutyl adducts to serve as substrates for other BER glycosylases has not been studied. Base excision repair is likely to play a role in repairing abasic sites generated by loss of 7-pobG or O^2-pobC.[19,49,50]

The lack of a clear relationship between a single pyridyloxobutyl DNA adduct and lung tumor formation differs from that observed with methylating agents, including NNK, in A/J mice.[8,16] In this latter case, strong correlations were observed between O^6-mG levels measured at 96 h and tumor multiplicity ($R^2 = 0.92$); 7-mG was not linked to tumor formation in this model. Consistently, mutations in the K-*ras* oncogene from these tumors were predominantly GC to AT transitions in the second base of codon 12.[14] GC to AT transition mutations are the primary mutation caused by methylating agents,[43] demonstrating the dominant role of O^6-mG in the overall mutagenic activity of these compounds. In contrast, NNKOAc induced mutations primarily at AT base pairs with less than 1/3 of the mutations occurred at GC base pairs in the *Hprt* gene of CHO cells.[19] Therefore, it is likely that pyridyloxobutylating agents are not as powerful at triggering mutations in the K-*ras* gene. Mutations in other oncogenes may be more critical for tumor formation by this alkylation pathway.

When our results are combined with those reporting the levels of methyl DNA adducts in NNK treated mice, a more complete understanding of the role of adducts in NNK-induced pulmonary tumorigenesis in A/J mice arises. The dominant mutagenic adduct formed in lungs from 10 μmol NNK-treated mice at either 8 h or 96 h post-treatment is O^6-mG; the levels of this adduct (30 ± 18 and 20 ± 5.1 pmol/μmol G, respectively)[8] are roughly four times the combined levels of the four pyridyloxobutyl DNA adducts (7.9 and 7.4 pmol/μmol dG, respectively, Table 1). This difference in mutagenic adduct levels likely explains why the methylation pathway makes a larger contribution to the overall tumorigenic activity of NNK in this animal model.[8,25,44]

In rats, the relative contribution of each pathway is less clear, with evidence supporting a major role for both.[7,9] DNA adduct studies have demonstrated that the levels of O^6-mG are similar to the levels of 7-pobG and less than or equal to the levels of O^2-pobdT following chronic exposure to 10 ppm NNK in the drinking water.[45] This is consistent with the available experimental evidence that indicates the mechanism of NNK-induced tumor formation is more complex in rats than in A/J mice.[1] It is also likely that different pyridyloxobutyl adducts are important in NNK-induced pulmonary tumorigenesis in rats since the relative distribution of the pyridyoxobutyl DNA adducts are quite different than what is observed in A/J mice. In rat lung, O^2-pobdT is the most abundant adduct with lower relative levels of 7-pobG and the levels of O^6-pobdG were 24–250 times lower than those of O^2-pobdT.[11] This observation suggests that there are species differences in pyridyloxobutyl DNA adduct formation and repair.

In summary, the findings from this study demonstrate that multiple adducts are likely to contribute to tumor formation from pyridyloxobutylating agents in A/J mice with 7-pobG, O^6-pobdG and O^2-pobdT all persisting at significant levels. Collectively, this study shows that tumor formation from the pyridyloxobutylating pathway of NNK is a complex process, one which likely involves multiple adducts and multiple repair pathways.

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Abbreviations

AGT	O^6-alkylguanine-DNA alkyltransferase
ANOVA	analysis of variance
BER	base excision repair
CHO	Chinese hamster ovary
dR	2'-deoxyribosyl
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
Fapy	6-diamino-4-hydroxy-5N-alkyl-formamidopyrimidine
HEPES	(4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid)
HPB	4-hydroxy-1-(3-pyridyl)-1-butanone
7-mG	7-methylguanine
NER	nucleotide excision repair
NNK	4-methylnitrosamino-1-(3-pyridyl)-1-butanone
NNKOAc	4-(acetoxymethylnitrosamino)-1-(3-pyridyl)-1-butanone
***O*^{4}-mT**	O^4-methylthymidine
***O*^{6}-bzG**	O^6-benzylguanine
***O*^{6}-mG**	O^6-methylguanine
PBS	phosphate buffered saline
PEG	polyethylene glycol
***O*^{2}-pobC**	O^2-pyriydloxobutylcytosine
***O*^{6}-pobdG**	O^6-pyridyloxobutylguanine
***O*^{2}-pobdT**	O^2-pyridyloxobutylthymidine
7-pobG	7-pyridyloxobutylguanine

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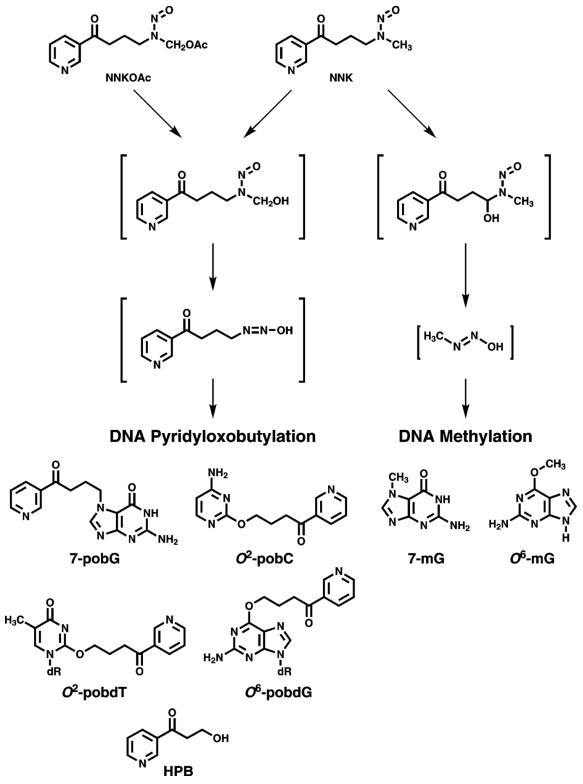

Figure 1.
Metabolic activation of NNK and the model pyridyloxobutylating agent NNKOAc.

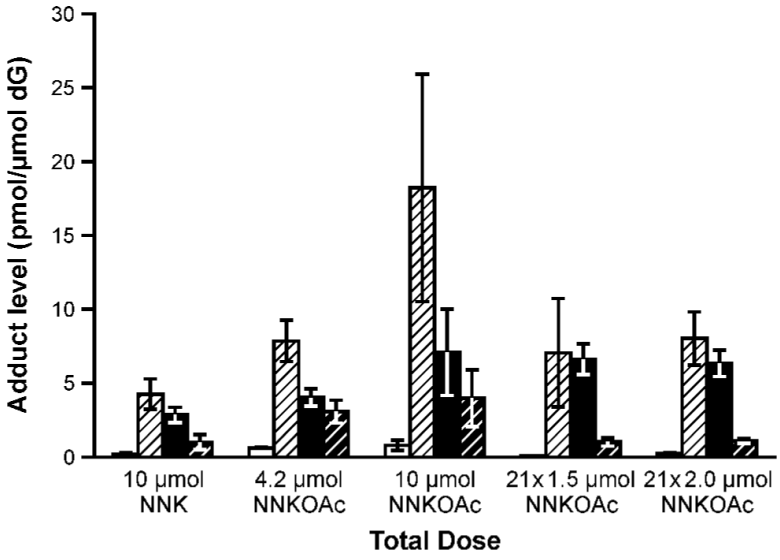

Figure 2.

Levels of pyridyloxobutyl DNA adducts in A/J mouse lungs 8 h following a single i.p. injection of 10 μmol NNK, 4.2 μmol or 10 μmol NNKOAc ± 2.5 μmol O^6-bzG or 8 h following the last of 21 i.p. injections of 1.5 μmol or 2.0 μmol NNKOAc ± 2.5 μmol O^6-bzG. O^2-pobC (□), 7-pobG (▨), O^2-pobdT (■), O^6-pobdG (◪).

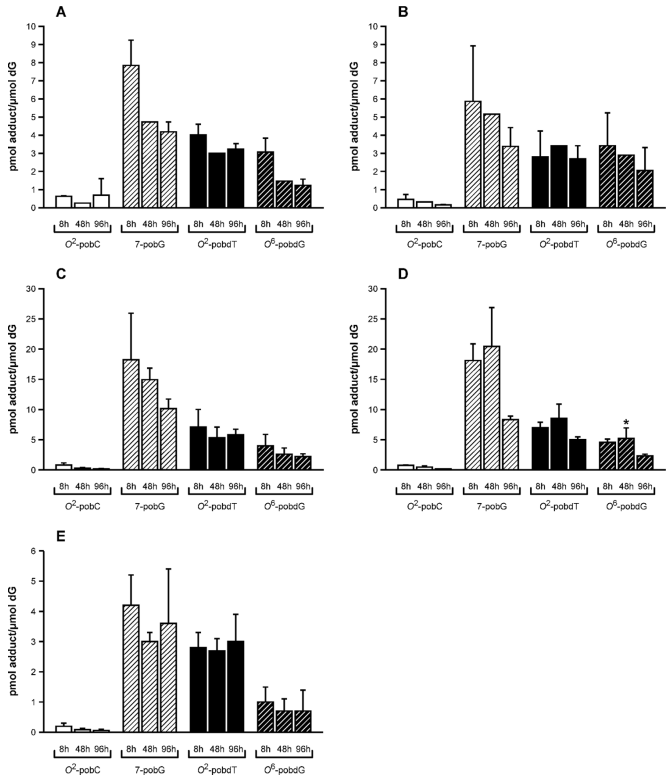

Figure 3.

Levels of pyridyloxobutyl DNA adducts in A/J mouse lungs 8, 48 or 96 h-post-treatment following a single injection 4.2 μmol NNKOAc (A and B) or 10 μmol NNKOAc (C and D) in the absence (A and C) or presence (B and D) of 2.5 μmol O^6-bzG or following a single injection of 10 μmol NNK (E). O^2-pobC (□), 7-pobG (▨), O^2-pobdT (■), O^6-pobdG (◪). *Significantly different from mice treated with the same concentration of NNKOAc without co-administration of 2.5 μmol O^6-bzG with Bonferroni adjustment, $P < 0.05$. **O^2-pobC significantly reduced with time, $P = 0.01$.

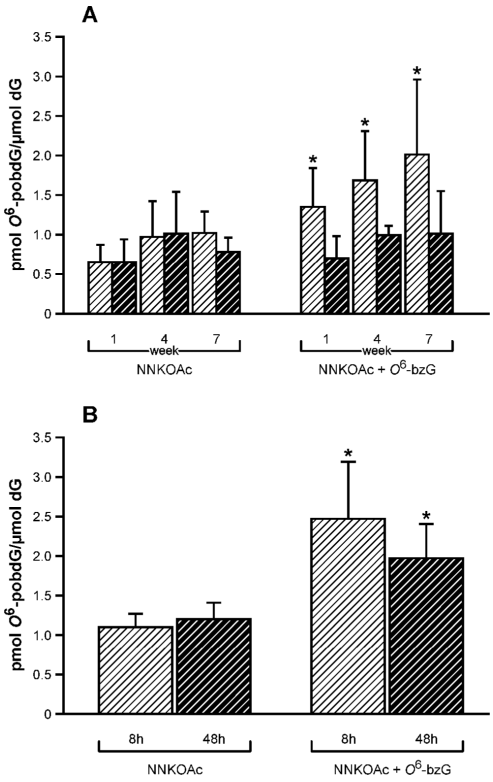

Figure 4.

Levels of O^6-pobdG in A/J mouse lung DNA 8 (▨) or 48 (◪) h following A) 3 (1 week), 12 (4 weeks) or 21 (7 weeks) injections of 1.5 μmol NNKOAc or B) 21 injections of 2.0 μmol NNKOAc. *Significantly different from mice treated with the same concentration of NNKOAc without co-administration of 2.5 μmol O^6-bzG with Bonferroni adjustment, $P < 0.05$.

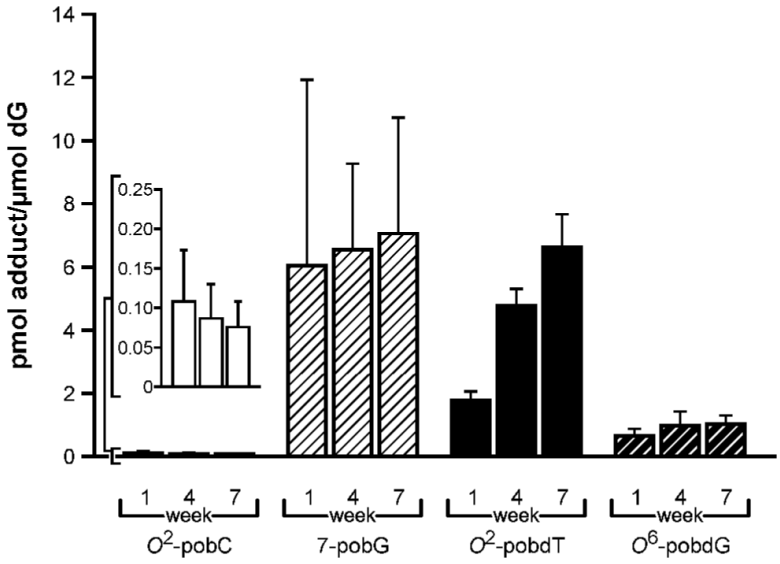

Figure 5.
Levels of pyridyloxobutyl DNA adducts in A/J mouse lungs 8 h-post-treatment following 3 (1 week), 12 (4 weeks) or 21 (7 weeks) injections of 1.5 μmol NNKOAc. O^2-pobC (□), 7-pobG (▨), O^2-pobdT (■), O^6-pobdG (◪).

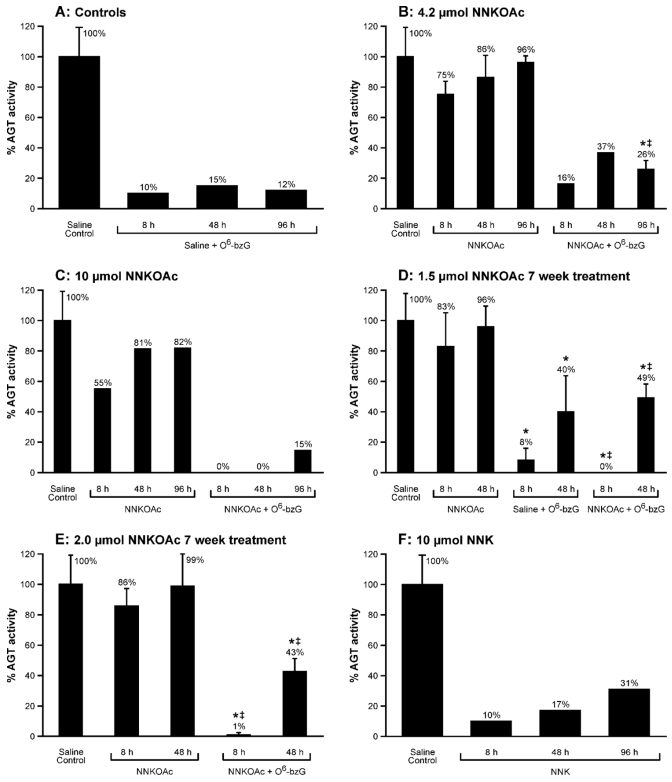

Figure 6.

AGT activity in mouse lungs 8, 48 or 96 h following treatment with compound relative to the activity in saline-treated control animals. A) O^6-bzG control animals receiving 4 daily doses of O^6-bzG; B) single dose of 4.2 μmol NNKOAc ± 4 daily doses of 2.5 μmol O^6-bzG; C) single dose of 10 μmol NNKOAc ± 4 daily doses of 2.5 μmol O^6-bzG; D) multiple doses of 1.5 μmol NNKOAc ± 2.5 μmol; E) multiple doses of 2.0 μmol NNKOAc ± 2.5 μmol O^6-bzG; F) single dose of 10 μmol NNK; The bars lacking error bars represent an average of two samples; the individual values of these samples were generally within 20% of each other. *Significantly different from saline control, $P \leq 0.05$; ‡Significantly different from NNKOAc alone, $P < 0.01$.

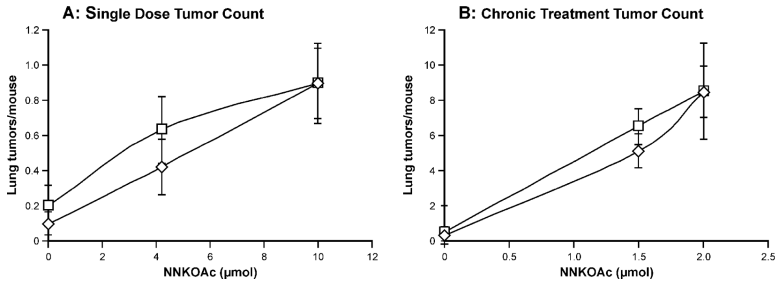

Figure 7.

Lung tumor multiplicity in A/J mice treated with single (A) or multiple (B) doses of NNKOAc ± O^6-bzG. Saline or 40% PEG-400 in phosphate buffered saline was substituted for the compounds in control groups. Minus O^6-bzG (◇), plus O^6-bzG (□).

Table 1

Pyridyloxobutyl DNA adducts observed following single and multiple dosing regimens of NNKOAc in the presence and absence of O^6-bzG.

	DNA Adduct Levels (pmol/umol dG ± SD)[a]											
	8 h				48 h				96 h			
Single Dose[b]	O^2-pobC	7-pobG	O^2-pobdT	O^2-pobdG	O^2-pobC	7-pobG	O^2-pobdT	O^6-pobdG	O^2-pobC	7-pobG	O^2-pobdT	O^6-pobdG
10 μmol NNK	0.2 ± 0.1	4.2 ± 1.0	2.8 ± 0.5	1.0 ± 0.5	0.09 ± 0.04	3.0 ± 0.3	2.7 ± 0.4	0.7 ± 0.4	0.06 ± 0.04	3.6 ± 1.8	3.0 ± 0.9	0.7 ± 0.2
4.2 μmol NNKOAc	0.6 ± 0.03[c]	7.9 ± 1.4[c]	4.0 ± 0.6[c]	3.1 ± 0.8[c]	0.3[d]	4.7[d]	3.0[d]	1.5[d]	0.7 ± 0.9[c]	4.2 ± 0.6[c]	3.2 ± 0.3[c]	1.2 ± 0.3[c]
4.2 μmol NNKOAc+O^6bzG	0.5 ± 0.3[c]	5.8 ± 3.1[c]	2.8 ± 1.4[c]	3.4 ± 1.8[c]	0.3[d]	5.2[d]	3.4[d]	2.9[d]	0.2 ± 0.0[c]	3.4 ± 1.0[c]	2.7 ± 0.7[c]	2.0 ± 1.3[c]
10 μmol NNKOAc	0.8 ± 0.4	18 ± 8	7.1 ± 3.0	4.0 ± 1.9	0.3 ± 0.1	15 ± 2	5.4 ± 1.7	2.6 ± 1.0	0.2 ± 0.0	10 ± 2	5.8 ± 0.9	2.2 ± 0.5
10 μmol NNKOAc+O^6bzG	0.8 ± 0.1	18 ± 3	7.0 ± 0.9	4.6 ± 0.5	0.5 ± 0.2	20 ± 6	8.5 ± 2.4	5.2 ± 1.7[e]	0.1 ± 0.0	8.3 ± 0.6	5.0 ± 0.5	2.3 ± 0.3
Chronic Dose[f]	1 week 8 h				4 weeks 8 h				7 weeks 8 h			
	O^2-pobC	7-pobG	O^2-pobdT	O^6-pobdG	O^2-pobC	7-pobG	O^2-pobdT	O^6-pobdG	O^2-pobC	7-pobG	O^2-pobdT	O^6-pobdG
Control	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
1.5 μmol NNKOAc	0.1 ± 0.1	6.1 ± 5.9	1.8 ± 0.3	0.7 ± 0.2	0.08 ± 0.04[g]	6.5 ± 2.7[g]	4.8 ± 0.5[g]	1.0 ± 0.5[g]	0.07 ± 0.03[g]	7.1 ± 3.7[g]	6.6 ± 1.0[g]	1.0 ± 0.3[g]
1.5 μmol NNKOAc+O^6bzG[i]	0.1 ± 0.1[g]	5.6 ± 3.1[g]	2.0 ± 0.7[g]	1.4 ± 0.5[e,g]	0.08 ± 0.06[g]	6.4 ± 3.3[g]	5.5 ± 1.3[g]	1.7 ± 0.6[e,g]	0.07 ± 0.06[c]	15 ± 17[c,h]	6.6 ± 0.2[c]	2.0 ± 1.0[c,e]
2.0 μmol NNKOAc	-	-	-	-	-	-	-	-	0.2 ± 0.0	8.0 ± 1.8	6.3 ± 0.9	1.1 ± 0.2
2.0 μmol NNKOAc+O^6bzG[i]	-	-	-	-	-	-	-	-	0.3 ± 0.0	11 ± 3	8.7 ± 2.3[h]	2.5 ± 0.7[e]
	1 week 48 h				4 weeks 48 h				7 weeks 48 h			
	O^2-pobC	7-pobG	O^2-pobdT	O^6pobdG	O^2-pobC	7-pobG	O^2-pobdT	O^6-pobdG	O^2-pobC	7-pobG	O^2-pobdT	O^6-pobdG
Control	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
1.5 μmol NNKOAc	0.06 ± 0.04	4.0 ± 2.3	1.9 ± 0.6	0.7 ± 0.3	0.05 ± 0.05[g]	6.8 ± 2.7[g]	5.7 ± 1.8[g]	1.0 ± 0.5[g]	0.04 ± 0.03	3.9 ± 2.6	6.5 ± 0.8	0.8 ± 0.2
1.5 μmol NNKOAc+O^6bzG	0.05 ± 0.04	3.8 ± 1.8	1.7 ± 0.4	0.7 ± 0.3	0.04 ± 0.03	6.3 ± 4.3	5.1 ± 1.0	1.0 ± 0.1	0.05 ± 0.03	7.7 ± 6.8	7.1 ± 0.6	1.0 ± 0.5
2.0 μmol NNKOAc	-	-	-	-	-	-	-	-	0.2 ± 0.0	7.9 ± 1.3	6.8 ± 0.9	1.2 ± 0.2
2.0 μmol NNKOAc+O^6bzG	-	-	-	-	-	-	-	-	0.2 ± 0.0	9.1 ± 1.2	7.8 ± 1.3	2.0 ± 0.4[e]

[a] Average of five samples ± SD except where noted. The levels of adducts ranged from 0–18 pmol/μmol dG, which is equivalent to 0 – 37 adducts/10^6 nucleotides.

[b] Groups of 15 mice were given a single i.p. injection of NNKOAc (4.2 or 10 μmol) or NNK (10 μmol). The NNKOAc-treated animals also received four daily i.p. injections of 40% PEG-400 in PBS or O^6-bzG in 40% PEG-400 in PBS with the first injection occurring immediate prior to the NNKOAc treatment. Saline was substituted for the compounds in control groups. Each group was subdivided into three groups for sacrifice at 8, 48, or 96 h following the nitrosamine treatment.

[c] Average of three samples ± SD.

[d] Average of two samples

[e] Significantly different from mice treated with the same concentration of NNKOAc without co-administration of 2.5 μmol O^6-bzG with Bonferroni adjustment, $P < 0.05$.

[f] Groups of 10 mice were given an i.p. injection 40% PEG-400 in PBS or O^6-bzG in 40% PEG-400 in PBS immediately followed by an i.p. injection of 1.5 or 2.0 μmol NNKOAc three times per week. Saline was substituted for the compounds in control groups. Each group was subdivided into two groups for sacrifice 8 or 48 h after the 3rd (1 week), 12th (4 weeks), or 21st (7 weeks) injections.

[g] Average of four samples ± SD.

[h] The number is an average of the following three values: 34, 5.1 and 6.0 pmol 7-pobG/Kmol dG.

[i] Levels of O^6-pobdG in NNKOAc-treated mice were significantly different from those receiving the same dose of NNKOAc without co-administration of 2.5 μmol O^6-bzG with ANOVA considering O^6-bzG presence, time and the interaction between them ($P < 0.01$).

Table 2

Lung tumor yields in A/J mice treated with NNKOAc in the presence or absence of O^6-bzG.

Treatment (μmol)	No O^6-bzG		+ 2.5 μmol O^6-bzG	
	% mice with tumors	Tumors/mouse Mean (± s.d.)	% mice with tumors	Tumors/mouse Mean (± s.d.)
Single Dose [a,c]				
Control	15.0[e]	0.1 ± 0.5	15.0[e]	0.2 ± 0.8
4.2 μmol NNKOAc	31.6[f]	0.4 ± 0.7	42.1[f]	0.6 ± 0.8
10 μmol NNKOAc	63.2[f]	0.9 ± 0.9	52.6[f]	0.9 ± 1.0
Chronic Dose [b,d]				
Control	23.5[g]	0.3 ± 0.5	42.1[f]	0.5 ± 0.6
21 × 1.5 μmol NNKOAc	100[h]	5.1 ± 3.1	100[e]	6.5 ± 2.9
21 × 2.0 μmol NNKOAc	100[f]	8.5 ± 2.9	100[e]	8.5 ± 3.8

[a] Groups of 20 mice were given a single i.p. injection of NNKOAc (4.2 or 10 μmol). Control animals received only saline. They also received four daily i.p. injections of 40% PEG-400 in PBS or O6-bzG in 40% PEG-400 in PBS with the first injection occurring immediate prior to the NNKOAc treatment. Animals were sacrificed 16 weeks after the first injection.

[b] Groups of 20 mice were given an i.p. injection 40% PEG-400 in PBS or O^6-bzG in 40% PEG-400 in PBS immediately followed by an i.p. injection of 1.5 or 2.0 μmol NNKOAc three times per week for 7 weeks. Saline was substituted for the compounds in control groups. Animals were sacrificed 35 weeks after the first injection.

[c] Two-way ANOVA indicated that the number of tumors per mouse were significantly with NNKOAc dose ($P < 0.01$, slope = 0.07 tumors/mouse/1 μmol).

[d] Two-way ANOVA indicated that the number of tumors per mouse were significantly with NNKOAc dose ($P < 0.01$, slope = 4.0 tumors/mouse/1 μmol).

[e] Based on 20 mice.

[f] Based on 19 mice.

[g] Based on 17 mice.

[h] Based on 18 mice.

Table 3

The ratio of 7-pobG levels to O^6-pobdG, O^2-pobdT or O^2-pobC levels 8 h following exposure to NNK or NNKOAc ± O^6-bzG[a]

	7-pobG/O^6-pobG	7-pobG/O^2-pobdT	7-pobG/O^2-pobC
NNKOAc *in vitro*[10]	1.6	2.8	3.6
NNK	4.2	1.5	20
4.2 μmol NNKOAc	2.6	2.0	12
4.2 μmol NNKOAc + O^6-bzG	1.7	2.1	13
10 μmol NNKOAc	4.6	2.6	23
10 μmol NNKOAc + O^6-bzG	4.0	2.6	24
3 × 1.5 μmol NNKOAc	9.3	3.4	61
12 × 1.5 μmol NNKOAc	6.7	1.4	82
21 × 1.5 μmol NNKOAc	6.9	1.1	100
3 × 1.5 μmol NNKOAc + O^6-bzG	4.1	2.8	50
12 × 1.5 μmol NNKOAc + O^6-bzG	3.8	1.2	81
21 × 1.5 μmol NNKOAc + O^6-bzG	7.6	2.3	220
21 × 2.0 μmol NNKOAc	7.3	1.3	33
21 × 2.0 μmol NNKOAc + O^6-bzG	4.3	1.2	42

[a]The adduct levels reported in Table 1 were used to determine the ratios reported in this table.