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Mass Spectrometric Quantitation of Pyridyloxobutyl DNA Phosphate Adducts in Rats Chronically Treated with *N*-Nitrosonornicotine

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Abstract

The tobacco-specific carcinogens *N*-nitrosonornicotine (NNN) and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) require metabolic activation to exert their carcinogenicity. NNN and NNK are metabolized to the same reactive diazonium ions, which alkylate DNA forming pyridyloxobutyl (POB) DNA base and phosphate adducts. We have characterized the formation of both POB DNA base and phosphate adducts in NNK-treated rats, and the formation of POB DNA base adducts in NNN-treated rats. However, POB DNA phosphate adducts in NNN-treated rats are still uncharacterized. In this study, we quantified the levels of POB DNA phosphate adducts in tissues of rats chronically treated with (*S*)-NNN or (*R*)-NNN for 10, 30, 50, and 70 weeks during a carcinogenicity study. The highest amounts of POB DNA phosphate adducts were observed in the esophagus of the (*S*)-NNN-treated rats, with a maximum level of 5400 ± 317 fmol/mg DNA at 50 weeks. The abundance of POB DNA phosphate adducts in the esophagus was consistent with the results of the carcinogenicity study showing that the esophagus was the primary site of tumor formation from treatment with (*S*)-NNN. Compared to the (*R*)-NNN group, the levels of POB DNA phosphate adducts were higher in the oral mucosa, esophagus and liver, while lower in the nasal mucosa of the (*S*)-NNN-treated rats. Among ten combinations of all isomers of POB DNA phosphate adducts, Ap(POB)C and combinations with thymidine predominated across all the rat tissues examined. In the primary target tissue, esophageal mucosa, Ap(POB)C accounted for ~20% of total phosphate adducts in the (*S*)-NNN treatment group throughout the 70 weeks, with levels ranging from 780 ± 194 to 1010 ± 700 fmol/mg DNA. The results of this study showed that POB DNA phosphate adducts were present in high levels and persisted in target tissues of rats chronically treated with (*S*)- or (*R*)-NNN. These results improve our understanding of DNA

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ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at www.acs.org: Figures S1–S3, Scheme S1, Tables S1–S8, and protocol for enzyme purification can be found in the supporting information.

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damage during NNN-induced carcinogenesis. The predominant POB DNA phosphate isomers observed, such as Ap(POB)C, may serve as biomarkers for monitoring chronic exposure of tobacco-specific nitrosamines in humans.

Graphical Abstract

INTRODUCTION

N-Nitrosornicotine (NNN, Scheme 1), one of the most abundant tobacco-specific nitrosamines present in cigarettes and smokeless tobacco, has been characterized as a powerful carcinogen in laboratory animals including rats, mice, hamsters, and mink.^{1–3} When administered in the drinking water, the esophagus and oral mucosa were the main target tissues in NNN-treated rats, while treatment by injection resulted mainly in tumors of the nasal mucosa. NNN is the main carcinogen in smokeless tobacco known to cause tumors of the oral mucosa, as seen in smokeless tobacco users. Furthermore, esophageal cancer risk was strongly associated with the urinary levels of NNN and its glucuronide in smokers from the Shanghai Cohort.⁴ NNN, together with the closely related tobacco-specific nitrosamine 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK, **3**), has been classified as a group 1 carcinogen by the International Agency for Research on Cancer.⁵

Due to the existence of a chiral carbon center at the 2'-position, two enantiomers of NNN, (*S*)-NNN (**1**) and (*R*)-NNN (**2**), can be formed. (*S*)-NNN, comprising an average of 63% of total NNN, was detected as the major enantiomer in U.S. tobacco products.⁶ A recent study showed that male F-344 rats had an average of 6.1 esophagus tumors and 4.5 oral cavity tumors per rat after chronic dosing of 14 ppm of (*S*)-NNN in the drinking water for 17 months, while (*R*)-NNN was less carcinogenic but significantly enhanced the activity of (*S*)-NNN.⁷

To exert their carcinogenicity, NNN and NNK need metabolic activation by cytochrome P450s.^{1–3} As illustrated in Scheme 1, both NNN enantiomers are oxidized through 2'-hydroxylation to form intermediate **5**. This is the preferential metabolism pathway leading to DNA adduct formation in rats, and is central to its mechanism of carcinogenicity.^{8–10} 5'-Hydroxylation also results in DNA adduct formation in rats, but to a lesser extent (Scheme S1).¹¹ The pyrrolidine ring of **5** opens up to form unstable diazohydroxide **8**, which decomposes to diazonium ion **10**. This highly reactive electrophile can pyridyloxobutylate DNA to form a panel of nucleobase adducts including compounds **13–16**, two of which can be converted to their stable forms **17** and **18** after neutral thermal hydrolysis (Figure 1).

Quantitation of these pyridyloxobutyl (POB) base adducts *in vitro* and *in vivo* indicates that **13** and **17** are the two major adducts, while the others were found in significantly lower levels.^{12–15} For NNK, α -methyl hydroxylation forms unstable compound **6** that also decomposes spontaneously to the same intermediate **8**. The major metabolite of NNK, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL, **4**),¹ undergoes a similar metabolism pathway to form pyridylhydroxybutyl (PHB) base adducts *in vivo* that have been well-characterized before (Scheme 1).^{16, 17}

While many studies have focused on DNA base adducts resulting from alkylating carcinogens, “DNA’s overlooked lesion” – phosphate adducts – have recently attracted more interest.¹⁸ DNA phosphate adducts are found in relatively high abundance, display long half-lives and are suggested to be less readily repaired when compared with related nucleobase alkylating products.^{19–21} One recent study suggested that the bacterial *O*⁶-alkylguanine transferase Ada was required for the TT to GT or GC mutations induced by the methyl DNA phosphate adduct lesion.²² Previous studies from our laboratory have shown that NNK-derived electrophilic diazonium ions **10–12** (Scheme 1) react with the nonbridging oxygens of the DNA internucleotide phosphate moiety.^{23–25} Both POB and PHB phosphate adducts were quantified in rat tissues at relatively high levels. Total DNA phosphate adducts (POB DNA phosphate adducts, PHB DNA phosphate adducts, together with methyl DNA phosphate adducts) represented 55–73% of all quantified DNA adducts in lung tissues from rats chronically treated with 5 ppm of NNK in the drinking water for 70 weeks.²⁶

Considering the convergence of the same reactive intermediate **8** generated from both NNN and NNK metabolism, it is reasonable to expect the formation of POB DNA phosphate adducts in tissues of NNN-treated rats. However, NNN and NNK have different metabolic fates in rat tissues, and the distribution pattern of POB DNA phosphate adducts in rats treated with NNN could be different from those following NNK treatment. Herein, we report the formation of POB DNA phosphate adducts in rats chronically treated with 14 ppm (*S*)-NNN or (*R*)-NNN in the drinking water for 70 weeks during a carcinogenicity study.^{7, 14} The adduct levels in the oral mucosa, esophageal mucosa, nasal respiratory mucosa, nasal olfactory mucosa, and liver were quantified using a highly sensitive and specific liquid chromatography-nano-electrospray ionization-high-resolution tandem mass spectrometry (LC-NSI-HRMS/MS) method.

EXPERIMENTAL DETAILS

Caution:

(*S*)-NNN and (*R*)-NNN are carcinogenic. They should be handled in a well-ventilated hood with extreme caution and with appropriate protective equipment.

Chemicals and supplies:

(*S*)-NNN and (*R*)-NNN were synthesized as reported previously.²⁷ The isotopically labelled internal standards [¹⁵N₃]Cp(POB)C and [¹³C₁₀¹⁵N₂]Tp(POB)T were available from our previous studies.^{23, 25} Cell lysis buffer (catalog number 158908), protein precipitation solution (catalog number 158912), proteinase K solution (catalog number 158920) and

RNase A solution (catalog number 158924) were purchased from Qiagen. Ribonuclease T solution (catalog number R1003–500KU) was purchased from Sigma-Aldrich. Deoxyribonuclease I (catalog number P4527–40KU), phosphodiesterase I (catalog number P3243–1VL) and alkaline phosphatase (Roche, catalog number 10567752001) were purchased from Sigma-Aldrich and purified before use (protocol described in the Supporting Information). All other chemicals and supplies were purchased from Sigma-Aldrich or Fisher Scientific. Milli-Q water (Millipore) was routinely used unless otherwise mentioned.

Animal Experiment:

This study was approved by the University of Minnesota Animal Care and Use Committee. The rats in this study were subgroups of the carcinogenicity study of the NNN enantiomers previously described.^{7,14} Briefly, male F-344 rats were treated with either (*R*)-NNN or (*S*)-NNN in their drinking water at a dose of 14 ppm for 70 weeks. The dosage of 14 ppm was based on our previous carcinogenicity studies of NNN in F-344 rats as well as estimated total human exposure to NNN.⁷ The rats in the (*S*)-NNN group began losing weight after 1 year and had died or were humanely euthanized by 17 months. The rats in the (*R*)-NNN group were terminated at 20 months based on decreasing weights.⁷ Six rats per group were humanely sacrificed by CO₂ overdose at 10, 30, 50, and 70 weeks.

Tissue harvest:

Tissues that were available from a previous study were retrieved as described.^{14, 15} Liver tissues were obtained from 3 rats at each time point. Nasal olfactory mucosa and respiratory mucosa in either the (*S*)-NNN group or the (*R*)-NNN group were obtained from 3 frozen rat heads at each time point. Oral mucosa – soft palate (for all samples) and surface layer of the front of the tongue (for all samples except 50 weeks and 70 weeks in the (*R*)-NNN group) – in each group were retrieved from the same 3 frozen heads as used for nasal mucosa retrieval at each time point. Esophageal mucosa in the (*S*)-NNN group were obtained from 3 frozen esophagi from the 30 weeks and 50 weeks groups. Only two esophagi in the (*S*)-NNN group were available from the 70 weeks group. No esophagi in the (*S*)-NNN group were available from the 10 weeks group. Esophageal mucosa in the (*R*)-NNN group were obtained from 3 frozen esophagi at each time point. All retrieved tissues were stored at –20 °C until DNA isolation.

DNA isolation and purification:

DNA was isolated from the animal tissues as described previously with a few modifications.^{14, 15} Briefly, 180–220 mg of oral mucosa, nasal olfactory mucosa, and nasal respiratory mucosa retrieved from each head were used for DNA isolation. About 20 mg of esophageal mucosa retrieved from each frozen esophagus were used for DNA isolation. About 500 mg of liver tissues were used for DNA isolation. The tissues were cut into small pieces, homogenized (except esophageal mucosa), and incubated with proteinase K overnight with gentle shaking at room temperature (for liver) or 37 °C (for the other tissues). RNase A and ribonuclease T were added the next day to the solution and incubated at 37 °C for 2 h. The protein precipitation solution was added and mixed by vortexing vigorously and then centrifuged at 4,000 *g* for 10 min at 4 °C. The supernatant was transferred into a clean tube containing ice-cold isopropanol. For the liver tissues, fluffy DNA started precipitating after

gentle inverting and swirling. The DNA was then transferred to a clean tube and washed with ice-cold 70% ethanol and 100% ethanol sequentially. The DNA was then dissolved in 5 mL of 10 mM Tris buffer containing 1 mM EDTA (pH = 7), and further purified by chloroform/isoamyl alcohol (24:1). The upper aqueous layer was carefully collected after centrifuging at 4,000 *g* for 15 min. The purification process was repeated twice. The collected aqueous solution was mixed with 0.5 mL of 5 M NaCl solution, followed by 8 mL of ice-cold isopropanol to precipitate the DNA. The DNA was then transferred to a clean vial and washed with ice-cold 70% ethanol and 100% ethanol sequentially; this was performed twice. DNA samples were dried under N₂ flow and stored at –20 °C for future use. For samples from other tissues, DNA was usually not visible unless centrifuged (15,000 *g* for 15 min) due to the small amounts of tissues being used. For esophageal mucosa samples, storing at –20 °C could help DNA precipitation upon centrifugation. After removal of the supernatant, the DNA was washed with ice-cold 70% ethanol and 100% ethanol sequentially. DNA samples were dried under N₂ flow and stored at –20 °C for future use.

DNA hydrolysis and sample preparation:

The same protocol was used as described previously.^{23–25} Briefly, the DNA sample (~200 µg) was dissolved in 600 µL of 10 mM sodium succinate buffer containing 5 mM CaCl₂ and then mixed with [¹⁵N₃]Cp(POB)C (10 fmol) and [¹³C₁₀¹⁵N₂]Tp(POB)T (5 fmol). The purified deoxyribonuclease I (0.8 units), phosphodiesterase I (5 milliunits) and alkaline phosphatase (2 units) were added and the mixture was incubated overnight with gentle shaking at 37 °C. On the next day, the solution was centrifuged at 13,000 *g* for 10 min. An aliquot of 20 µL supernatant was taken for dGuo quantitation by HPLC and determination of DNA amount considering that dGuo comprises 22% of all nucleotides in rat DNA (Supplementary Table S1). The remaining hydrolysate was filtered through 10K centrifugal filters (Millipore, Microcon-10, catalog number MRCPRT010). The filtrate was loaded to a 30 mg Strata-X SPE cartridge (Phenomenex, catalog number 8B-S100-TAK) that was activated with 2 mL MeOH and preconditioned with 2 mL H₂O. The cartridge was washed with 2 mL of H₂O and 1 mL of 10% MeOH before the analyte was eluted with 2 mL of 50% MeOH. The 50% MeOH fractions were concentrated to dryness and redissolved in 15 µL of 2% MeOH solution (Fisher, Optima MeOH and Optima H₂O, catalog numbers A456–4 and W6–4) prior to mass spectrometric analysis.

LC-NSI-HRMS/MS analysis:

POB DNA phosphate adducts were analyzed using a previously developed method with a few modifications.^{23–25} Briefly, an aliquot of 3 µL was injected into a RSLCnano system equipped with a 5 µL injection loop. Separation was performed with a nanoLC column (50 µm i.d., 360 µm o.d., 17–22 cm length and 15 µm orifice) hand-packed with 5 µm Luna C₁₈ bonded separation media (Phenomenex, Torrance, CA). The gradient was slightly adjusted to afford an improved chromatographic separation for all isomers (Table S2). Samples were analyzed by nano-electrospray using an Orbitrap Fusion detector with full scan, selected-ion monitoring (SIM) and product ion scan analysis. A multiplexed SIM analysis was performed using an isolation window of 1.5 *m/z*. The accurate mass tolerance used for extraction of precursor and fragment ion signals was 5 ppm. Quantitative analysis of all phosphate adducts was performed as reported.^{23, 24} Briefly, the relative signal intensities of the major

fragment ions of both synthetic native DNA adduct standards and isotopically labelled internal standards were compared. The instrument response and the Cp(POB)C/[¹⁵N₃]Cp(POB)C ratio were linear in the 0.15–15 fmol range. Synthesized standard Tp(POB)T behaved similarly as Cp(POB)C.

LC-NSI-HRMS/MS segment method:

The segment method was created from the LC-NSI-HRMS/MS method described above. Instead of detecting all 10 possible combinations throughout the run in the SIM mode, a method that can detect fewer numbers of combinations in short time periods was established. Since different combinations of POB DNA phosphate adducts eluted on the column with different retention times, segmenting the run time to different time periods, e.g., 23.8–26.5 min, 29.6–33.0 min, would allow the Orbitrap Fusion detector to focus on fewer numbers of target compounds, consequently improving the sensitivity. This method required a preliminary run to determine the retention times of all target compounds. Time segmentation was then adjusted accordingly.

Statistical analysis:

Levels of POB DNA phosphate adducts, POB DNA base adducts, and total quantified POB DNA adducts were summarized with means and standard deviations. One-way analysis of variance (ANOVA) was used to compare adduct levels between the four time points for each type of tissue of rats chronically treated with (*S*)-NNN or (*R*)-NNN. The subgroup comparisons of adduct levels between the time points were adjusted by the Bonferroni method. In all statistical tests, a p-value of 0.05 was considered significant. All statistical analyses were implemented using SAS statistical software, version 9.4 (SAS Institute Inc., Cary, NC, USA.).

RESULTS

Two non-carbon-bonded oxygen atoms exist in each DNA phosphate backbone moiety. Alkylation, by interacting with carcinogens or their metabolically activated products (such as NNN in this study), on either oxygen will yield phosphotriesters with two configurations of the phosphorus atom, namely *R*_p and *S*_p.¹⁸ As shown before, POB DNA phosphate adducts existed in the form of B₁p(POB)B₂ after enzyme hydrolysis due to their resistance to the applied conditions.²³ Since B₁ and B₂ represent the same or different nucleobases, there will be 10 combinations of DNA phosphate adducts, with a total of 32 possible structurally unique DNA phosphate adducts including stereoisomers being formed through pyridyloxobutylation by the NNN metabolite.

Characterization of POB DNA Phosphate Adducts

The previously described method that had been used for analyzing DNA phosphate adducts was modified, with the aim of observing all 32 possible stereoisomers.^{23–25} Improved chromatographic separation and detection sensitivity were achieved by using a thinner column (50 μm i.d. Luna C₁₈ column) and a shallower linear gradient at a slower flow rate (Table S2). All 32 isomers were observed in rat esophageal mucosa and nasal mucosa (Table S3). Since phosphate adduct levels in the livers from the (*R*)-NNN treatment were below the

detection limit, a segment method with improved sensitivity (with more isomers observed, Table S3) was applied. However, levels of POB DNA phosphate adducts in these liver samples were still unquantifiable.

Representative chromatograms of the two isotopically labelled internal standards [$^{15}\text{N}_3$]Cp(POB)C and [$^{13}\text{C}_{10}^{15}\text{N}_2$]Tp(POB)T were essentially the same as previously reported.²³ Corresponding adduct peaks coeluted at the same retention times as their internal standards (Figure S1). Typical chromatograms of selected-ion-monitoring (SIM) and major fragment ions of one of the combinations – Ap(POB)C – were also similar to those previously reported (Figure S2). The abundance pattern of product ions of Ap(POB)C²³ together with its fragmentation pattern suggested that the fragment ion m/z 326.0788 (loss of one nucleotide and one base plus one H_2O from precursor ions) may serve as a diagnostic ion for phosphate adduct characterization. The extracted chromatograms of transitions from parent compounds to the fragment ion m/z 326.0788 of all combinations are shown in Figure 2. There were only three stereoisomers (one isomer each of Tp(POB)T, Cp(POB)T and Gp(POB)T) not visible in this transition mode. However, they could be observed by checking other fragment ion transitions. Since each combination contains two or four isomers, it is clearly shown in Figure 2 that these isomers were observed to various extents.

With two synthesized internal standards in hand, Cp(POB)C and Tp(POB)T were quantified by stable isotope dilution.²⁴ For other adducts with no standards available, amounts were estimated using their SIM signal intensities compared to Cp(POB)C or Tp(POB)T. The data obtained was then averaged for the comparisons discussed below. Observations of relative mass signal intensities of various standards were also consistent with our previous report.²⁴

POB DNA Phosphate Adduct Levels in the Rat Oral Mucosa

All 10 combinations of the POB DNA phosphate adducts were detected in the oral mucosa of rats treated with either (*S*)-NNN or (*R*)-NNN, with observed total numbers of isomers being 29 or 24, respectively (Table S3). Total levels of POB DNA phosphate adducts in the rats treated with (*S*)-NNN decreased throughout the 50 weeks and then increased to the maximum level of 290 ± 156 fmol/mg DNA at the 70-week time point (Figure 3A, Table 1). In the (*R*)-NNN treatment group, the levels were relatively stable ranging from 30 ± 15 to 70 ± 48 fmol/mg DNA. Phosphate adduct levels in the (*S*)-NNN group were 3.5–6.7 fold higher than those in the (*R*)-NNN group (statistically significant at 10, 30, and 70 weeks, Table S4). The adduct levels of the 10 individual combinations showed the same trend with the (*S*)-NNN group having higher levels of phosphate adducts than the (*R*)-NNN group (Figure S3A). The most predominant combination was Gp(POB)T, which accounted for 21–33% of total POB DNA phosphate adducts in the (*S*)-NNN group and 19–45% in the (*R*)-NNN group (Table S5). Cp(POB)T and Tp(POB)T also showed relatively high levels in both groups.

POB DNA base adduct levels in the oral mucosa of rats from the same animal experiment had been quantified previously.¹⁴ The levels of POB DNA phosphate adducts were significantly lower than those of the base adducts in both the (*S*)-NNN and (*R*)-NNN groups (Table 1, Table S6). In the (*S*)-NNN group, phosphate adduct levels were 2.3–6.9 fold lower than base adduct levels. In the (*R*)-NNN group, phosphate adduct levels were 3.7–8.4 fold

lower than base adduct levels. For total DNA adduct levels, both groups showed similar increasing trends throughout the experiment, with statistical significance observed in the (*R*)-NNN group ($p = 0.049$) (Figure 4A). Phosphate adducts represented 30% of all quantified POB DNA adducts at week 10 in the oral mucosa of rats treated with (*S*)-NNN, and 21% at week 30, 13% at week 50, 24% at week 70 (Table S7). In the (*R*)-NNN group, the numbers were slightly lower: 17% at week 10, 21% at week 30, 11% at week 50, and 18% at week 70.

POB DNA Phosphate Adduct Levels in the Rat Esophageal Mucosa

All 32 isomers of POB DNA phosphate adducts were detected in the esophageal mucosa from rats treated with (*S*)-NNN or (*R*)-NNN. Total levels of POB DNA phosphate adducts in the (*S*)-NNN group showed an increasing trend with a maximum level of 5400 ± 317 fmol/mg DNA at 50–70 weeks (Figure 3B, Table 1). The phosphate adduct level was relatively stable in the (*R*)-NNN group throughout the experiment, ranging from 970 ± 211 to 2110 ± 1268 fmol/mg DNA. The 1.9–5.6 fold differences between the two groups were significant at 50- and 70-weeks (Table S4). Distribution patterns of combinations were similar to the oral mucosa, in which (*S*)-NNN treatment had significantly higher levels of phosphate adducts than (*R*)-NNN for each combination (Figure S3B). The three highest levels of combinations were Ap(POB)C, Ap(POB)T, and Cp(POB)T. They accounted for 19–20%, 17–22%, 17–22% or 14–19%, 18–23%, 20–22% of total phosphate adducts in the (*S*)-NNN or (*R*)-NNN group, respectively (Table S5).

When compared with POB base adducts, higher levels of phosphate adducts were observed by 1.6–2.1 or 1.0–2.9 fold in the (*S*)-NNN or (*R*)-NNN groups, respectively (Table 1). However, there were no statistically significant differences except for the 10-week data in the (*R*)-NNN group (Table S6). The total quantified DNA adduct levels of the (*S*)-NNN group were marginally increased over time but with a p -value of 0.23 (Figure 4B). There was no clear trend for the (*R*)-NNN group. Of total DNA adducts in the esophageal mucosa, POB phosphate adducts represented surprisingly 58–68% in the (*S*)-NNN group and 51–75% in the (*R*)-NNN group (Table S7).

POB DNA Phosphate Adduct Levels in the Rat Nasal Respiratory Mucosa

Similar to the rat esophageal mucosa, all 32 isomers were detected in the nasal respiratory mucosa from both groups. Levels of the POB DNA phosphate adducts from either (*S*)-NNN or (*R*)-NNN treatment showed negligible increasing trends ($p = 0.47$ for the (*S*)-NNN group, 0.12 for the (*R*)-NNN group) throughout the time course of this study (Figure 3C, Table 1). POB DNA phosphate adduct levels ranged from 1720 ± 156 to 2160 ± 495 fmol/mg DNA in the (*S*)-NNN group and from 2100 ± 327 to 3680 ± 934 fmol/mg DNA in the (*R*)-NNN group. Adduct levels in the (*R*)-NNN group were marginally but significantly higher (1.6–2.1 fold) than the (*S*)-NNN group at the 10-, 30- and 70-week time points (Table S4). Consistent with the patterns of total POB phosphate adducts, slightly higher levels of phosphate adducts in the combinations were found in the nasal respiratory mucosa from rats treated with (*R*)-NNN (Figure S3C). The four most frequently occurring combinations Ap(POB)C, Ap(POB)T, Cp(POB)T, and Gp(POB)T together represented 61–66% of the POB DNA phosphate adducts in both groups (Table S5).

POB DNA base adducts in the nasal respiratory mucosa determined previously were at fairly high levels (Table 1). POB base adducts were significantly 3.2–4.2 fold higher than phosphate adducts in the (*S*)-NNN group, but only 1.0–1.8 fold higher in the (*R*)-NNN group (not significant) (Table S6). Total DNA adduct levels of both groups showed increasing trends throughout the experiment but with no statistical significance ($p = 0.33$ for the (*S*)-NNN group, 0.16 for the (*R*)-NNN group) (Figure 4C). The percentages of phosphate adducts of total DNA adducts varied in the nasal respiratory mucosa: 19–24% were observed in the (*S*)-NNN group, while 36–50% were observed in the (*R*)-NNN group (Table S5).

POB DNA Phosphate Adduct Levels in the Rat Nasal Olfactory Mucosa

The nasal olfactory mucosa results were similar to those of the nasal respiratory mucosa. All isomers were detected in the (*R*)-NNN group; only one isomer of Cp(POB)G was missing in the (*S*)-NNN group (Table S3). Treatment with (*S*)-NNN showed an increasing trend with the maximum level of 250 ± 87 fmol/mg DNA reached at 70 weeks (Figure 3D, Table 1). The adduct levels from the (*R*)-NNN treatment showed a rapidly increasing trend with the maximum level of 670 ± 121 fmol/mg DNA achieved at 30 weeks and relatively stable levels of ~ 520 fmol/mg DNA at 50 and 70 weeks. The levels of phosphate adducts in the (*R*)-NNN group were 2.1–4.0 fold significantly higher than the (*S*)-NNN group at all time points except 10 weeks (Table S4). The POB DNA phosphate adduct levels of ten combinations had a similar distribution pattern as in the nasal respiratory mucosa. The (*R*)-NNN group had higher levels of phosphate adducts than the (*S*)-NNN group in each combination (Figure S3D). Gp(POB)T was the most abundant combination in the (*R*)-NNN group but showed a significantly decreasing trend after 30 weeks ($p = 0.03$). The other three predominant combinations Ap(POB)C, Cp(POB)T, and Tp(POB)T remained relatively stable throughout the study. These four combinations together accounted for 54–60% or 55–63% of total phosphate adducts in the (*S*)- or (*R*)-NNN group, respectively (Table S5).

POB phosphate adduct levels were significantly lower than base adduct levels in both the (*S*)-NNN and (*R*)-NNN groups (Table 1, Table S6). In the (*S*)-NNN group, they were 2.8–4.1 fold lower than base adducts, representing 20–26% of total quantified DNA adducts (Table S7). In the (*R*)-NNN group, phosphate adduct levels were 2.8–5.1 fold lower, representing 16–26% of total adducts. Total DNA adduct levels showed significantly increasing trends ($p = 0.002$ or 0.02 for the (*S*)-NNN or (*R*)-NNN treatment, respectively) in both groups (Figure 4D). The levels of total DNA adducts in the (*R*)-NNN group were 3.4–3.9 fold higher than in the (*S*)-NNN group, a significant difference (Table 1, Table S4).

POB DNA Phosphate Adduct Levels in the Rat Liver

Twenty-two isomers of POB DNA phosphate adducts were detected in the liver samples from the (*S*)-NNN treatment but only 6 in the liver samples from the (*R*)-NNN treatment (Table S3). The levels decreased significantly from 410 ± 84 fmol/mg DNA at 30 weeks to ~ 200 fmol/mg DNA at 70 weeks in the (*S*)-NNN treatment group ($p = 0.02$) (Figure 3E, Table 1). For (*R*)-NNN, the levels of most POB phosphate adducts were below the detection limit. Gp(POB)T was the most predominant combination in the liver samples of rats treated with (*S*)-NNN (Figure S3E). Its level was relatively stable, ranging from 50 ± 20 to 70 ± 38

fmol/mg DNA throughout the experiment. Gp(POB)T accounted for 12–31% of all liver POB DNA phosphate adducts in this group. Three other abundant combinations were Ap(POB)C, Cp(POB)T, and Ap(POB)T. These four combinations together represented 67–85% of POB DNA phosphate adducts detected in the liver tissues from the (*S*)-NNN treatment (Table S5).

POB DNA base adduct levels were also relatively low in the liver samples from the (*R*)-NNN treatment. However, the base adduct levels in the (*S*)-NNN group were 1.7–3.2 fold higher than the phosphate adducts, a significant difference (Table 1, Table S6). Total levels of quantified DNA adducts showed a clear decreasing trend throughout the study ($p = 0.007$) (Figure 4E). The percentage of POB DNA phosphate adducts was 24–37% of total POB DNA adducts in the (*S*)-NNN group (Table S7).

DISCUSSION

This is the first study to identify DNA phosphate adducts resulting from NNN treatment. Using a highly sensitive and specific LC-NSI-HRMS/MS method, we were able to identify up to the maximum 32 unique DNA phosphate adducts which could be formed by 2'-hydroxylation of the NNN enantiomers in each of 5 tissues from rats treated with these carcinogens for 10, 30, 50, or 70 weeks. These rats were part of a study that demonstrated the potent carcinogenicity of (*S*)-NNN (14 ppm in the drinking water) in the rat oral and esophageal mucosa, while (*R*)-NNN was less active.⁷ Taken together with a previous study of DNA base adducts formed from NNN in the same rats,¹⁴ the results present a unique and comprehensive picture of DNA damage by this tobacco-specific carcinogen.

The highest levels of POB DNA phosphate adducts were observed in the esophageal mucosa of the rats treated with (*S*)-NNN. They also accounted for the highest proportions, reaching 58–68%, of total quantified DNA adducts. This is consistent with previous studies which demonstrate that the rat esophagus has high activity for the metabolism of (*S*)-NNN by 2'-hydroxylation resulting in relatively high levels of DNA base adducts in this tissue.^{12, 14, 15} It is also consistent with the effect on the esophagus of (*S*)-NNN observed in the parallel study of NNN carcinogenicity carried out under the same conditions.⁷ In that study, a total of 122 esophageal tumors were observed in the 20 rats treated with (*S*)-NNN. Although the levels of DNA phosphate adducts in the esophagus were not statistically higher than the DNA base adducts in this tissue, their relative amounts were nevertheless remarkable, with the most abundant phosphate adducts Ap(POB)C, Ap(POB)T and Cp(POB)T persisting throughout the study at levels of 770–1160 fmol/mg DNA. These results suggest that DNA phosphate adducts should be further investigated with regard to their ability to cause mutations and contribute to carcinogenicity in the rat esophagus, a common target tissue of nitrosamines including NNN. We note however that POB phosphate adduct levels as well as base adduct levels were relatively lower in the oral mucosa than the esophagus and nasal mucosa even though the oral mucosa was a major target tissue in the carcinogenicity assay, with a total of 89 tumors being observed in the 20 rats treated with (*S*)-NNN in the drinking water. This was likely due to the fact that the retrieved oral mucosa was not a uniform mucosa layer, unlike the esophagus. Separation of the mucosa layer from connective tissues

including muscle was difficult from frozen rat heads. Furthermore, the relatively high turnover rate of oral mucosa cells could also dilute the detected DNA adduct levels.

High levels of POB DNA phosphate adducts and POB DNA base adducts were observed in the nasal mucosa, both respiratory and olfactory. This is consistent with previous studies that have demonstrated extensive metabolic activation of NNN in cultured rat nasal mucosa and abundance of cytochrome P450 2A3, an efficient catalyst of NNN 2'-hydroxylation, in the rat olfactory mucosa. The relatively high levels of POB DNA phosphate adducts in the nasal mucosa were mostly consistent with previous POB base adduct analysis results.¹³⁻¹⁵ In the present study, in both the respiratory mucosa and olfactory mucosa, (*R*)-NNN treatment caused higher levels of DNA phosphate adducts than (*S*)-NNN treatment. In the olfactory mucosa, POB DNA base adduct levels were also higher in the (*R*)-NNN group when compared to the (*S*)-NNN group. This is likely because of the hepatic first pass effect since the carcinogens were administered in the drinking water. After preferential metabolism of (*S*)-NNN, the amount of (*R*)-NNN exceeded its counterpart resulting in a higher accumulation of (*R*)-NNN in the nasal mucosa.²⁸ Extensive 2'-hydroxylation consequently caused a similar pattern of corresponding DNA adduct distribution due to the presence of abundant P450s in the rat nose.^{29, 30} However, the reversed pattern of POB DNA base adduct levels in the nasal respiratory mucosa reported by Zhao et al¹⁴ is incompatible with this proposed explanation. Experimental error in that study has been excluded and the observation remains unexplained. Although high levels of DNA adducts were observed in the rat nasal mucosa, tumors occurred mainly in the esophagus and oral mucosa under the conditions of this study in which the NNN enantiomers were administered in the drinking water. Studies of NNN administered by subcutaneous injection to rats have uniformly produced mainly nasal tumors, consistent with the high levels of metabolic activation in this tissue.¹ We have proposed that the oral and esophageal tumors produced by oral administration of NNN are lethal and kill the rats before nasal tumors can develop.

Different efficiencies of repair mechanisms of POB phosphate adducts versus base adducts likely contribute to their relative levels in various tissues. The presence of *O*⁶-alkylguanine DNA alkyltransferase, base excision repair, and nucleotide excision repair affect the persistence of POB base adducts **13–16** (Figure 1).^{31, 32} However, POB DNA phosphate adducts were considered to be refractory to repair in mammalian cells. The half-lives of methyl or ethyl DNA phosphate adducts were 7 days or 32 days in rats receiving a single intraperitoneal injection of dimethylnitrosamine or *N*-ethyl-*N*-nitrosourea.²¹ Ethyl DNA phosphate adducts also persisted in mouse liver, with ~37% and ~15% of the initial adducts remaining after 4 days and 56 days treatment, respectively.³³ The bacterial Ada protein can stereospecifically repair methyl DNA phosphate adducts in *Escherichia coli*^{34, 35} but no repair mechanisms for DNA phosphate adducts have been reported in eukaryotic systems.¹⁸ Taken together, repair efficiency likely plays an important role in preserving high levels of different DNA adducts in rat tissues, but further studies are required.

Levels of each combination of POB DNA phosphate adducts were compared. Ap(POB)C and combinations with thymidine including Ap(POB)T, Cp(POB)T, Gp(POB)T, and Tp(POB)T generally had the highest persistence across all five tissues. This distribution pattern was similar to that found in our study of POB DNA phosphate adducts formed from

NNK via the common intermediate **8** (Scheme 1), except for the combination Tp(POB)T.²³ The exceptionally high levels of Tp(POB)T observed previously were likely due to its high mass signal intensities since no internal standard for this adduct was available for quantitation at that time.²⁴ Most of the combination levels had an increasing or stable trend throughout the experiment, with the exception of Gp(POB)T in the nasal olfactory mucosa and a few combinations in the liver. The proportions of certain combinations of POB DNA phosphate adducts were also relatively stable at high levels. For example, Ap(POB)C was detected as the most abundant combination in the esophageal mucosa. It accounted for 20%, 19%, and 20% of total phosphate adducts at 30, 50, and 70 weeks in the (*S*)-NNN treatment group. Similarly, Ap(POB)C accounted for 17%, 18%, 17%, and 19% at the four time points in the nasal respiratory mucosa from rats treated with (*R*)-NNN. With respect to their abundance and long-term persistence, certain POB DNA phosphate adducts may serve as reliable bio-markers for chronic alkylating carcinogen exposure. However, internal standards are required for absolute quantitation of specific combinations.

Taking Ap(POB)C as an example, there were four stereoisomers observed in Figure S2. Their proportions were clearly not the same, with two being more abundant. The persistence level of each isomer likely reflects the balance of occurrence and repair efficiency. Although evidence showing that the *S_p* isomer could be repaired by the Ada protein while the *R_p* isomer was retained among methyl DNA phosphate adducts, it is not clear how this mechanism would affect POB phosphate adduct levels in mammalian cell lines.^{34, 35} Another study in *Escherichia coli* cells showed that the *S_p* isomer of methyl DNA phosphate adducts could be efficiently bypassed and induced TT to GT or GC mutations with the requirement of Ada. On the other hand, the *R_p* isomer could moderately suppress DNA replication.²² This study along with previous observations suggested that stereoisomers exert distinct biological effects with different mutagenic or carcinogenic properties.³⁶ However, with no authentic synthesized standards in hand, stereochemical aspects of each peak in Figure S2 remain unclear. Work is in progress on the synthesis of relevant standards to address these questions.

Only 2'-hydroxylation products of NNN were considered in this study. As depicted in Scheme S1, both NNN enantiomers can also be metabolically activated through the 5'-hydroxylation pathway.¹ POB DNA phosphate adducts represented 58–68% or 51–75% of total DNA adducts from 2'-hydroxylation in the esophageal mucosa in either the (*S*)- or (*R*)-NNN treatment group. However, the proportions were much lower in the other tissues. For example, only 13–30% or 11–21% in the oral mucosa and 20–26% or 16–26% in the nasal olfactory mucosa from rats treated with (*S*)- or (*R*)-NNN respectively were observed. This difference can be partially explained since 5'-hydroxylation of NNN occurred minimally in the rat esophagus but to a higher extent in the rat nasal cavity under the catalysis of cytochrome P450 2A3.^{37, 38} Thus it is likely that new phosphate adducts are formed through the NNN 5'-hydroxylation pathway. A previous study from our lab identified 2-(2-(3-pyridyl)-*N*-pyrrolidinyl)-2'-deoxyinosine as the major 5'-hydroxylated DNA base adduct formed from NNN in rats. However, its levels were significantly lower than POB base adducts in rat tissues.¹¹ In spite of its relatively low level, exploration of phosphate adducts resulting from 5'-hydroxylation of NNN would still be interesting, especially when

considering the fact that 5'-hydroxylation occurred to a higher extent than 2'-hydroxylation in the patas monkey and certain human tissues.³⁹⁻⁴⁴

In summary, we report a comprehensive quantitation of POB DNA phosphate adducts in five tissues from rats chronically treated with either (*S*)-NNN or (*R*)-NNN using a highly sensitive and specific LC-NSI-HRMS/MS method. Levels of these POB DNA phosphate adducts were particularly high in the esophageal mucosa, a target tissue of NNN carcinogenicity. The abundance of predominant combinations of POB DNA phosphate adducts was consistent with our previous study of DNA phosphate adducts formed from NNK. These data lead to a better understanding of the complex pattern of DNA damage induced by NNN during chronic treatment leading to tumor formation in rats. The predominant combinations of DNA phosphate adducts observed in this study, e.g. Ap(POB)C, may serve as useful biomarkers for monitoring chronic exposure to tobacco-specific nitrosamines in humans who use tobacco products.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS

NNN	<i>N</i> '-Nitrosornicotine
NNK	4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone
POB	pyridyloxobutyl
NNAL	4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol
PHB	pyridylhydroxybutyl
LC-NSI-HRMS/MS	liquid chromatography-nano-electrospray ionization-high-resolution tandem mass spectrometry
EDTA	ethylenediaminetetraacetic acid
HPLC	high performance liquid chromatography
SPE	solid phase extraction
i.d.	inner diameter

o.d.	outer diameter
SIM	selected ion mode

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Figure 1.
Structures of representative POB DNA base adducts, dR = 2'-deoxyribose.

Figure 2.

Representative chromatograms of fragment ion m/z 326.0788 of all possible POB DNA phosphate adducts.^a

^a This data is from one sample of DNA from the rat nasal respiratory mucosa of rats treated with (*R*)-NNN for 50 weeks.

Figure 3. Plots of total POB DNA phosphate adduct levels (fmol/mg DNA) vs time (weeks) in the (A) oral mucosa, (B) esophageal mucosa, (C) nasal respiratory mucosa, (D) nasal olfactory mucosa, and (E) liver from rats chronically treated with (S)-NNN or (R)-NNN.

Figure 4.

Plots of total quantified DNA adduct levels resulting from 2'-hydroxylation of NNN (fmol/mg DNA) vs time (weeks) in the (A) oral mucosa, (B) esophageal mucosa, (C) nasal respiratory mucosa, (D) nasal olfactory mucosa, and (E) liver from rats chronically treated with (S)-NNN or (R)-NNN.

Scheme 1.

Overview of pyridyloxobutyl (POB) and pyridylhydroxybutyl (PHB) DNA adduct formation from NNN, NNK, and NNAL.

Table 1.

Levels of POB DNA phosphate adducts, POB DNA base adducts and total quantified POB DNA adducts in the five tissues from rats chronically treated with (S)-NNN or (R)-NNN.

Tissue	Time (weeks)	(S)-NNN			(R)-NNN		
		Phosphate adducts (fmol/mg DNA)	Base adducts ^a (fmol/mg DNA)	Total adducts (fmol/mg DNA)	Phosphate adducts (fmol/mg DNA)	Base adducts ^a (fmol/mg DNA)	Total adducts (fmol/mg DNA)
Oral mucosa	10	260 ± 60	590	850 ± 60	40 ± 10	180	220 ± 10
	30	200 ± 30	750	950 ± 30	60 ± 54	210	270 ± 54
	50	120 ± 27	850	980 ± 27	30 ± 15	210	230 ± 15
	70	290 ± 156	920	1210 ± 156	70 ± 48	330	400 ± 48
Esophageal mucosa	10	NA ^b	2030	NA ^b	1400 ± 229	740	2140 ± 229
	30	3910 ± 715	2440	6350 ± 715	2110 ± 1270	720	2830 ± 1270
	50	5400 ± 317	2550	7950 ± 317	970 ± 211	950	1920 ± 211
	70	4970 ^c	3660	8630	990 ± 246	700	1690 ± 246
Nasal respiratory mucosa	10	1740 ± 206	6720	8460 ± 206	2830 ± 355	3250	6080 ± 355
	30	1720 ± 156	6800	8520 ± 156	3550 ± 720	4060	7610 ± 720
	50	2160 ± 495	6860	9010 ± 495	2100 ± 327	3720	5820 ± 327
	70	1910 ± 198	8080	10000 ± 198	3680 ± 934	3720	7400 ± 934
Nasal olfactory mucosa	10	150 ± 48	430	580 ± 48	340 ± 74	1700	2030 ± 74
	30	170 ± 41	590	760 ± 41	670 ± 121	1890	2560 ± 121
	50	150 ± 7	600	750 ± 7	520 ± 121	1850	2370 ± 121
	70	250 ± 87	720	970 ± 87	520 ± 151	2160	2680 ± 151
Liver	10	340 ± 59	730 ± 83	1060 ± 102	< LOD	120 ± 12	120 ± 12
	30	410 ± 84	690 ± 61	1100 ± 104	< LOD	110 ± 26	1130 ± 26
	50	190 ± 38	460 ± 48	660 ± 61	< LOD	90 ± 10	90 ± 10
	70	200 ± 50	630 ± 99	820 ± 111	< LOD	80 ± 11	80 ± 11

Numbers in this table were rounded.

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^aData are from Zhao, et al. *Chem. Res. Toxicol.* 2013, 26, 1526–1535; two samples were quantified in rat tissues except for liver ($N=4$).

^bNA: not available.

^cTwo samples were quantified due to experimental material availability.