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Identification of Adducts Formed in the Reactions of 5'-Acetoxy-N'-nitrososornicotine with Deoxyadenosine, Thymidine, and DNA

Pramod Upadhyaya and Stephen S. Hecht*

Masonic Cancer Center, University of Minnesota Minneapolis, MN 55455

Abstract

N'-Nitrososornicotine (NNN) is the most prevalent of the carcinogenic tobacco-specific nitrosamines found in all tobacco products. Previous studies have demonstrated that cytochrome-P450 mediated 5'-hydroxylation of NNN is a major metabolic pathway leading to mutagenic products, but to date DNA adducts formed by this pathway have been only partially characterized, and there have been no studies reported on adducts formed with bases other than dGuo. Since adducts with dAdo and dThd have been identified in DNA of the livers of rats treated with the structurally related carcinogen N-nitrosopyrrolidine, we investigated dAdo and dThd adduct formation from 5'-acetoxyNNN (**3**), a stable precursor to 5'-hydroxyNNN (**2**). Reaction of **3** with dAdo gave diastereomeric products which were identified by their spectral properties and LC-ESI-MS/MS-SRM analysis as N⁶-[5-(3-pyridyl) tetrahydrofuran-2-yl]dAdo (**9**). This adduct was further characterized by NaBH₃CN reduction to N⁶-[4-hydroxy-4-(3-pyridyl)but-1-yl]dAdo (**17**). A second dAdo adduct was identified, after NaBH₃CN treatment, as 6-[2-(3-pyridyl)pyrrolidin-1-yl]purine-2'-deoxyriboside (**18**). Reaction of **3** with dThd, followed by NaBH₃CN reduction, gave O²-[4-(3-pyridyl)-4-hydroxybut-1-yl]thymidine (**11**). Adducts **9**, **11**, **17**, and **18** were all identified by LC-ESI-MS/MS-SRM comparison to synthetic standards. The reaction of **3** with calf thymus DNA was then investigated. The DNA was enzymatically hydrolyzed to deoxyribonucleosides and the resulting mixture was treated with NaBH₃CN and analyzed by LC-ESI-MS/MS-SRM. Adducts **11**, **17**, and **18**, as well as the previously identified dGuo adducts, were identified. The results of this study provide a more comprehensive picture of DNA adduct formation by the quantitatively important 5'-hydroxylation pathway of NNN and will facilitate investigation of the presence of these adducts in laboratory animals treated with NNN or in people who use tobacco products.

Keywords

N'-nitrososornicotine; DNA adducts

Introduction

N'-Nitrososornicotine (NNN, **1**, Scheme 1), a tobacco-specific nitrosamine, is one of the most prevalent strong carcinogens in tobacco products (1,2). It occurs in parts per million levels in unburned tobacco including moist snuff that is consumed orally, as well as in the mainstream and sidestream smoke of cigarettes, generally in amounts of hundreds of nanograms per cigarette (3). NNN causes tumors of the esophagus and nasal cavity in rats, and tumors of the respiratory tract in hamsters, mice, and mink (2). A mixture of NNN and the related nitrosamine 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) caused oral tumors in rats when swabbed in the oral cavity (4). NNN and NNK are carcinogenic to humans, according to a

*To whom correspondence should be addressed: Masonic Cancer Center, University of Minnesota, MMC 806, 420 Delaware St SE, Minneapolis, MN 55455, USA. phone: (612) 626-7604 fax: (612) 626-5135 e-mail: hecht002@umn.edu.

working group of the International Agency for Research on Cancer (3). In three recent genome wide association studies (5-7), variants in nicotine acetylcholine receptor subunit genes were strongly associated with lung cancer, and NNN - receptor interactions were cited as one possible mechanism (5).

NNN requires metabolic activation by cytochrome P450 catalyzed 2'- and 5'-hydroxylation to express its mutagenic and presumably its carcinogenic activity (3,8). As shown in Scheme 1, 5'-hydroxylation results in the formation of 5'-hydroxyNNN (**2**), an unstable compound which spontaneously ring opens to electrophiles such as **4** - **6**. These intermediates react with DNA to form adducts which are critical in the carcinogenic process. Our goal is to develop a comprehensive picture of DNA adduct formation from NNN, which will ultimately lead to a better understanding of the mechanisms by which it causes cancer and possibly to rational approaches to cancer prevention in people who are exposed to this tobacco-specific carcinogen. Toward this goal, we have previously characterized a variety of DNA adducts produced by 2'-hydroxylation of NNN, such as 7-[4-(3-pyridyl)-4-oxobut-1-yl]dGuo and *O*²-[4-(3-pyridyl)-4-oxobut-1-yl]thymidine (**9**), as well as dGuo/DNA adducts (**7**, **10**, **12** and **15**, Scheme 1) formed by 5'-hydroxylation (**10**). In this study, we extended our work on DNA adducts of NNN by further characterizing adducts resulting from 5'-hydroxylation. 5'-HydroxyNNN (**2**) was generated by esterase treatment of 5'-acetoxyNNN (**3**) in the presence of dAdo, dThd, and DNA, and the resulting adducts were characterized by their spectral properties and by comparison to synthetic standards. We focused on dAdo and dThd because previous studies of the related cyclic nitrosamine *N*-nitrosopyrrolidine, which lacks a pyridine ring, have demonstrated adduct formation with these two bases in vitro and in vivo (11,12).

Materials and Methods

Caution

NNN and 5'-acetoxyNNN are carcinogenic and mutagenic and therefore should be handled with extreme care, using appropriate clothing and ventilation at all times.

Chemicals and Enzymes

These were obtained from Sigma-Aldrich Chemical Co. (Milwaukee, WI), unless noted otherwise. 5'-AcetoxyNNN (**3**) and *O*²-[4-(3-pyridyl)-4-hydroxybut-1-yl]thymidine (**11**) were synthesized as previously described (13,14).

Apparatus and Assay Conditions

HPLC was carried out with Waters Associates (Milford, MA) systems equipped with a model 996 photodiode array detector or a model 440 UV-visible detector set to 254 nm, unless noted otherwise. The following solvent elution programs were used with linear solvent gradients: System 1, a 300 × 3.9 mm, 10 μm C-18 Bondclone column (Phenomenex, Torrance, CA) eluted at 1 mL/min with 0 - 100% CH₃OH in 15 mM NH₄OAc (pH 5.5) over the course of 60 min; System 2, a 250 mm × 10 mm, 10 μm C-18 Vydac 201TP column (Separations Group, Hesperia, CA) eluted at 3 mL/min with 5 - 40% CH₃CN in 15 mM NH₄OAc (pH 5.5) over the course of 40 min. LC-ESI-MS/MS was carried out in systems 3-5 as follows. System 3 used an Agilent 1100 series capillary flow LC/MSD trap (Agilent Technologies, Palo Alto, CA) with a 150 mm × 0.5 mm Zorbax Extend-C-18 5 μm column eluted at 10 μL/min with 5% CH₃OH in 15 mM NH₄OAc (pH 5.5), held for 10 min, then increased to 95 % CH₃OH over the course of 60 min. N₂ was used as the drying gas (200 °C, 5 L/min) and as nebulizing gas (15 psi). The system also contained an in line UV detector. The mass spectrometer was operated in the full scan mode (*m/z* 100-800), with target - ion abundance of 30 000, maximum accumulation time of 300 ms, and fragmentation amplitude of 0.9 V. MS/MS experiments were performed with a CID gas pressure of 1.5 mTorr and CID energy of 30 V. System 4 was the same as System

3 except the column was eluted at 15 $\mu\text{L}/\text{min}$ with 5% CH_3CN in 15 mM NH_4OAc (pH 5.5) to 65% CH_3CN over the course of 40 min. System 5 was the same as System 3 except the HPLC was interfaced with a TSQ Quantum Ultra instrument (Thermo Finnigan LCMS Division, San Jose, CA). The ESI source was set in the positive mode as follows: voltage, 4kV; current, 20 μA ; and ion transfer tube, 150 $^\circ\text{C}$. MS/MS experiments used the selected reaction monitoring (SRM) mode. The collision energy was 13 eV, and the argon collision gas pressure was 0.8 mTorr. High-resolution mass spectra (HRMS) were determined with a Finnigan-MAT 9095 instrument (Thermo Finnigan MAT, Bremen, Germany). NMR spectra were recorded in $\text{DMSO}-d_6$ using Varian Unity (Varian, Inc., Palo Alto, CA) spectrometers operated at either 600 or 800 MHz and standard 5 mm tubes or 3 mm Shigemi tubes (Shigemi, Inc., Allison Park, PA).

Reaction of 5'-acetoxyNNN (3) with dAdo or dThd

5'-AcetoxyNNN (**3**, 4.59 mg, 0.02 mmol) was allowed to react with dAdo (12.6 mg, 0.05 mmol) or dThd (12.0 mg, 0.05 mmol) in 5 mL of 0.1M phosphate buffer, pH 7.0, in the presence of porcine liver esterase (90 units) at 37 $^\circ\text{C}$ for 1 h. Adduct **9** was characterized directly, and adducts **11**, **17** and **18** after NaBH_3CN treatment (40 mg, 37 $^\circ\text{C}$, 1h). Analysis was by LC-ESIMS/MS-SRM using systems 3 and 5 and by comparison to synthetic standards.

Reaction of 5'-acetoxyNNN (3) with DNA

5'-AcetoxyNNN (**3**, 12.0 mg, 0.05 mmol) was allowed to react with calf thymus DNA (20 mg) in 5 mL of 0.1 M phosphate buffer, pH 7.0, in the presence of porcine liver esterase (90 units) at 37 $^\circ\text{C}$ for 1 h. The DNA was precipitated by addition of ethanol, then washed with 70% and 95% ethanol. The DNA (1.2 mg) was dissolved in 1 mL of 10 mM Tris HCl/5 mM MgCl_2 buffer, pH 7.0. NaBH_3CN (10 mg) was added and the mixture was kept at 37 $^\circ\text{C}$ for 30 min, and the DNA was then hydrolyzed enzymatically (10). The hydrolysate was subjected to solid phase extraction on C18 Sep-pak cartridges (Waters) as described (10). Products **11**, **12**, **15**, **17** and **18** were eluted with CH_3OH and analyzed by LC-ESI-MS/MS-SRM using system 5 and by comparison to synthetic standards.

6-[2-(3-Pyridyl)pyrrolidin-1-yl]purine-2'-deoxyriboside (18)

Nornicotine (**23**, Scheme 3, 1.8 mg, 0.012 mmol) and 6-chloropurine-2'-deoxyriboside (**22**, 6.5 mg, 0.024 mmol) were dissolved in DMSO (600 μL) containing Et_3N (100 μL) and the mixture was heated at 60 $^\circ\text{C}$ for 12 h. The solvent was partially removed under reduced pressure and the product was purified by HPLC using system 1, starting from 10% MeOH , retention time 32.2 min (0.760 mg, 0.002 mmol, 17%). ^1H NMR ($\text{DMSO}-d_6$) δ : 8.44 [unk]br, 1H, pyr-2H), 8.37 (br, 1H, pyr-6H), 8.18 (s, 1H, H8), 8.04 (s, 1H, H2), 7.54 (br, 1H, pyr-4H), 7.26 (dd, $J=5.0, 7.0\text{Hz}$, 1H, pyr-5H), 6.31 (br, 1H, H1'), 6.19 (br, 0.5H, pyrr-2aH), 5.50 (br, 0.5H, pyrr-2bH), 5.28 (br, 1H, 3'-OH), 5.12 (br, 1H, 5'-OH), 4.40 (br, 0.5H, pyrr-5Ha), 4.37 (br, 1H, H3'), 4.21 (br, 0.5H, pyrr-5Hb), 4.04 (br, 0.5H, pyrr-5Hc), 3.84 (br, 1H, H4'a), 3.80 (br, 0.5H, pyrr-5Hd), 3.56 (br, 1H, H5'a), 3.49 (br, 1H, H5'b), 2.66 (br, 1H, H2'a), 2.41 (br, 1H, pyrr-3Ha), 2.23 (br, 1H, H2'b), 2.02-1.80 (m, 3H, pyrr-3Hb and pyrr-4H). UV ($\text{CH}_3\text{OH}/15\text{mM}$ NH_4OAc) λ_{max} 212.4, 275.0. LCESI-MS/MS and HPLC-UV analysis were performed using system 4, retention time 18.6 min; MS m/z 383 $[\text{M} + \text{H}]^+$; MS/MS of m/z 383; m/z 267; SRM analysis (m/z 383 $\rightarrow$ m/z 267) was carried out using system 5, retention time 45.1 min; HRMS calcd for $\text{C}_{19}\text{H}_{22}\text{N}_6\text{O}_3$, 382.470, found, 382.175.

N⁶-[5-(3-Pyridyl)tetrahydrofuran-2-yl]dAdo (9)

5-(3-Pyridyl)-2-hydroxytetrahydrofuran (**19**) (5.8 mg, 0.035 mmol) was dissolved in 0.5 mL of dry DMF under N_2 . Trifluoroacetic acid (2 μL) and dAdo (8.8 mg, 0.035 mmol) were added, and the mixture was allowed to stir at room temperature for 4 days. Reaction progress was

monitored by HPLC using system 1. Retention times were as follows for diastereomers of **9**: 33.4; 33.48; 35.12; 35.51 min). Material eluting from 33–37 min was collected and the solvent was removed to give **9** (3.2 mg, 0.008 mmol, 22%). ¹H NMR (DMSO-*d*₆) δ : 8.55 [unk]s, 1H, pyr-2H), 8.47 (d, *J* = 4.2 Hz, 1H, pyr-6H), 8.44 (br, 1H, NH), 8.43 (s, 1H, dAdo-H8), 8.28 (s, 1H, dAdo-H2), 7.75 (dd, *J* = 6.0, 7.8 Hz, 1H, pyr-4H), 7.36 (dd, *J* = 4.8, 7.8 Hz, 0.5H, pyr-5Ha), 7.32 (dd, *J* = 5.4, 7.8 Hz, 0.5H, pyr-5Hb), 6.37 (t, *J* = 6.6 Hz, 1H, H1'), 5.30 (d, *J* = 3.6 Hz, 1H, 3'-OH), 5.14 (t, *J* = 6.0 Hz, 1H, 5'-OH), 5.08 (t, *J* = 6.6 Hz, 1H, THF-2H), 4.96 (t, *J* = 6.6 Hz, 1H, THF-5H), 4.41 (br, 1H, H3'), 3.88 (br, 1H, H4'), 3.60 (m, 1H, H5'a), 3.52 (m, 1H, H5'b), 2.72 (m, 1H, H2'a), 2.52 (m, 1H, THF-3H), 2.35 (m, 1H, THF-4Ha), 2.27 (m, 1H, H2'b), 2.11 (m, 1H, THF-4Hb), 1.77 (m, 1H, THF-3Hb). UV (CH₃OH/15mM NH₄OAc) λ_{max} peaks 1 and 3, 213.4, 263.9 nm, peaks 2 and 4, 213.4, 265.0 nm. LC-ESI-MS/MS and HPLC-UV analysis were carried out using system 3. Retention times were: peaks 1 and 2, 41.9; peak 3, 44.1; peak 4, 44.2 min. MS *m/z* 399 (*M* + *H*)⁺; MS/MS of *m/z* 399; *m/z* 283; SRM analysis (*m/z* 399 → *m/z* 283) was performed using system 5, retention time 39.8 min; HRMS calcd for C₁₉H₂₂NaN₆O₄, 421.1595 (*M* + *Na*)⁺; found, 421.1598.

N⁶-[5-(*S*)-(3-pyridyl)tetrahydrofuran-2-yl]dAdo (9**)**

5-(*S*)-**19** (10) (0.18 mg, 0.0011 mmol) was allowed to react with dAdo (0.31 mg, 0.0011 mmol) in DMF (0.3 mL) and trifluoroacetic acid (0.5 mL) for 4 days at room temperature, as described above. (*S*)-**9** was purified using HPLC system 1, retention time 41.8 and 44.1 min.

N⁶-[4-Hydroxy-4-(3-pyridyl)but-1-yl]dAdo (17**)**

A mixture of racemic adduct **9** (1.1 mg, 0.003 mmol) and NaBH₃CN (10 mg) in CH₃OH / H₂O (1:1) was stirred overnight at room temperature. The pH was adjusted to 7.0 with 0.1N HCl, and **17** was purified using HPLC system 1, yielding 0.6 mg (50%), retention time 32.8 min. ¹H NMR (DMSO-*d*₆) δ : 8.49 [unk]s, 1H, pyr-2H), 8.41 (d, *J* = 4.2 Hz, 1H, pyr-6H), 8.30 (s, 1H, dAdo-H8), 8.17 (s, 1H, dAdo-H2), 7.86 (br, 1H, NH), 7.69 (d, *J* = 7.8 Hz, 1H, pyr-4H), 7.31 (dd, *J* = 5.4, 7.8 Hz, 1H, pyr-5H), 6.33 (t, *J* = 7.2 Hz, 1H, H1'), 5.31 (br, 1H, 4-OH), 5.29 (br, 1H, 3'-OH), 5.23 (br, 1H, 5'-OH), 4.61 (br, 1H, H4), 4.39 (br, 1H, H3'), 3.86 (br, 1H, H4'), 3.60 (m, 1H, H5'a), 3.51 (m, 1H, H5'b), 3.47 (m, 2H, H1), 2.71 (m, 1H, H2'a), 2.23 (m, 1H, H2'b), 1.65 (m, 3H, H3 + H2a), 1.55 (m, 1H, H2b). UV (CH₃OH/15mM NH₄OAc) λ_{max} 213.4, 265.0. LC-ESI-MS/MS and HPLC-UV analysis were done using system 4, retention time 14.2 min; MS *m/z* 401 [*M* + *H*]⁺; MS/MS of *m/z* 401; *m/z* 285; SRM analysis (*m/z* 401 → *m/z* 285) was carried out using system 5, retention time 39.8 min; HRMS calcd for C₁₉H₂₄NaN₆O₄, 423.1751 [*M* + *Na*]⁺; found, 423.1756.

Adduct **17** was also prepared by treating **21** (Scheme 2, 0.5 mg, 0.0012 mmol), prepared essentially as described for the corresponding N²-dGuo adduct (10), with NaBH₄ (5 mg) in CH₃OH (3 mL). The HPLC retention time, UV, MS (system 4), and ¹H NMR spectra of **17** prepared in this way matched those of **17** prepared from **9** using system 2.

Results

Reaction of **3** with dAdo followed by LC-ESI-MS/MS-SRM analysis at *m/z* 399 → *m/z* 283 produced the chromatogram illustrated in Figure 1. The UV spectrum of peak 1 is shown in Figure 2; the spectra of peaks 2–4 were essentially identical to it and all are characteristic of N⁶-substituted dAdo derivatives. Since diastereomers of adduct **10** are formed by the reaction of the exocyclic amino group of dGuo with intermediate **6** (Scheme 1), these data suggested that the observed peaks were diastereomers of **9**, resulting from the reaction of the exocyclic amino group of dAdo with **6**. Standard adduct **9** was prepared by reaction of 5-(3-pyridyl)-2-hydroxytetrahydrofuran (**19**) with dAdo.

LC-ESI-MS/MS-SRM analysis of the product produced 4 main peaks similar to those eluting from 40-46 min in Figure 1, and their UV spectra were the same as that shown in Figure 2. The $^1\text{H-NMR}$ spectrum of the mixture was completely consistent with the overall structural assignment as **9**.

Further evidence for the structural assignment of **9** was obtained by NaBH_3CN treatment of this adduct, expected to be in equilibrium with Schiff base **14** (Scheme 1). This produced **17**, which was identical to a standard prepared by the reaction of keto aldehyde **20** with dAdo in the presence of NaBH_3CN to give **21**, followed by NaBH_4 reduction (Scheme 2). Adduct **17** matched the product obtained upon NaBH_3CN treatment of the reaction mixture of **3** and dAdo, as established by LC-ESI-MS-SIM at m/z 401 $[\text{M} + \text{H}]^+$, and by LC-ESI-MS/MS-SRM [m/z 401 $\rightarrow$ m/z 285] (Figure 3A,B). These results conclusively established the overall structure of adduct **9**.

The stereochemistry of **9** was then investigated by allowing 5-(*S*)-**19**, which we had previously synthesized (**10**), to react with dAdo giving adducts **9** with known absolute configuration at

the tetrahydrofuran carbon (carbon 5, see Figure 1) bearing the pyridine ring. Using these standards, we established that peaks 1 and 3 of Figure 1 are the 5-(*S*)-diastereomers while peaks 2 and 4 are the 5-(*R*)-diastereomers. Peaks 1 and 3 were interconvertible, as were peaks 2 and 4. Thus, collection of peak 3 from HPLC system 1 and re-injection within 2 h at room temperature produced a mixture of peaks 1 and 3. These results are consistent with the equilibrium involving Schiff base **14**, as shown in Scheme 1, limiting our ability to determine the absolute configuration at the 2-position. Similar results were previously observed with dGuo adduct **10**, Scheme 1 (10).

While adduct **13** (Scheme 1) was an expected product of the reaction of 5'-acetoxyNNN (**3**) with dAdo, by analogy to the dGuo reactions which produced adduct **7**, we found no evidence for this product by LC-ESI-MS/MS-SRM at m/z 399 $\rightarrow$ m/z 283, possibly due to the instability of this adduct which most likely exists in equilibrium with **8** and **16**. However, when the reaction mixture of 5'-acetoxyNNN and dAdo was treated with NaBH₃CN, adduct **18** (Scheme 1) was produced cleanly, based on LC-ESI-MS/MS-SRM (m/z 383 $\rightarrow$ m/z 267) (Figure 4A, B). It was identified by comparison to standard **18**, which was prepared as shown in Scheme 3.

Reaction of dThd with 5'-acetoxyNNN (**3**) followed by LC-ESI-MS/MS-SRM analysis at m/z 390 $\rightarrow$ m/z 274, which corresponds to the expected fragmentation involving loss of deoxyribose from potential adduct **24**, did not produce any reproducible peaks. However, treatment of this reaction mixture with NaBH₃CN did yield a single peak when analyzed by LCESI-MS/MS-SRM for m/z 392 $\rightarrow$ m/z 276 (Figure 5A), corresponding to an adduct with possible structure **11** (Scheme 1), which had been previously synthesized in connection with our studies of pyridylhydroxybutylation of DNA by 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (13,14). Standard **11** was co-injected with the product of the NaBH₃CN reaction and the mixture analyzed by LC-ESI-MS/MS-SRM. This product co-eluted with standard **11**, as illustrated in Figure 5B, confirming its identity.

The formation of adducts **17**, **18**, and **11**, as well as the previously characterized dGuo adducts **12** and **15** (Scheme 1), in DNA that was reacted with 5'-acetoxyNNN, and then treated with NaBH₃CN, was investigated. Enzymatic hydrolysates of this DNA were analyzed by LCESI-MS/MS-SRM, as illustrated in Figure 6. The results provided clear evidence for the presence of each adduct by comparison to standards analyzed under the same conditions. In separate analyses of each adduct, similar results were obtained (data not shown). Although quantitation was not attempted, it appears that the dGuo adducts **12** and **15** are formed in the greatest amounts.

Discussion

Metabolic 5'-hydroxylation of NNN was identified as a mutagenic pathway three decades ago (15), but little information is available on the DNA adducts that are formed. We have recently shown that dGuo adducts **7** and **10**, as well as **12** and **15** (after reduction), are formed from the

carbocation and oxonium ion pathways, respectively, as illustrated in Scheme 1 (10). In this study, we have extended our investigation of DNA adduct formation by these pathways, demonstrating that 5'-hydroxylation of NNN also leads to adducts with dAdo and dThd. Convincing evidence for the presence of these adducts in reaction mixtures containing 5'-acetoxyNNN, esterase, and the appropriate deoxyribonucleosides or DNA, was obtained by LCESI-MS/MS-SRM analysis in comparison to synthetic standards.

The dAdo adducts are quite analogous to the dGuo adducts previously characterized, involving initial attack by the exocyclic amino group of dAdo on either **4** and **5** (Scheme 1) or on oxonium ion **6** or a related intermediate. While this might have been considered an expected result, particularly in view of the fact that α -acetoxy-*N*-nitrosopyrrolidine (**25**) forms structurally similar dAdo adducts (11), we note that

these are the first examples of dAdo adducts originating from tobacco-specific nitrosamines, with the exception of the formaldehyde-derived DNA adducts of 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK), which do not incorporate the pyridyloxobutyl group (16). The exocyclic amino group of dAdo may be more reactive with the electrophiles generated here from 5'-hydroxylation of NNN than with the pyridyloxobutylating agent **27** formed via **26** by methyl hydroxylation of NNK or via **28** by 2'-hydroxylation of NNN (Scheme 4).

In our previous study of the reactions of **25** with dThd and DNA, we observed adduct formation, but the adducts could not be fully characterized due to their instability (11). Treatment of these reaction mixtures with NaBH₃CN did however produce the stable adducts **29** and **30**, which were characterized by their spectral properties and by comparison to independently synthesized standards. Similarly, in this study, we could not observe stable dThd products resulting from the reaction of 5'-acetoxyNNN with dThd or DNA, but adduct **11** was produced upon

NaBH₃CN reduction of these mixtures. We cannot exclude the possibility that *O*⁴-substituted-dThd adducts or dThd adducts derived from intermediates **4** and **5** of the carbocation pathway may also be formed. Further research on dThd adducts may be warranted if in vivo studies indicate that adduct **11** is quantitatively important, and similar considerations apply to dCyd adducts. *O*²-(POB-1-yl)dThd, resulting from 2'-hydroxylation of NNN, is present in significant quantities in the esophagus of NNN-treated rats (9), and adducts **29** and **30** have been observed in NaBH₃CN-treated hepatic DNA of rats treated with *N*-nitrosopyrrolidine (12).

A study by Kanki et al in Sprague-Dawley gpt δ rats treated with *N*-nitrosopyrrolidine in the drinking water demonstrated a 10 fold increase in mutant frequency compared to controls (17). The main type of base substitution observed was an A:T to G:C transition and this was the only change observed more frequently than in controls. These results suggest the possible importance of *N*-nitrosopyrrolidine adducts with dAdo or Thd, and, considering their structural similarities, similar effects might be observed upon treatment with NNN, but we are not aware of any published studies of specific mutations induced by NNN in animals.

As only the second most potent carcinogen among the tobacco-specific nitrosamines (2), NNN has received less attention than NNK, and this difference has been accentuated by the fairly extensive use of the NNAL biomarker to indicate NNK uptake in people exposed to tobacco products (18,19), while parallel biomarker research on NNN has only commenced fairly recently (20,21). Yet levels of NNN are consistently higher than those of NNK in tobacco products (3), and the carcinogenicity of NNN, while weaker than that of NNK, is nevertheless notable. A comparative dose-response study of NNN and NNK in rats demonstrated that they induce a comparable and high incidence of nasal tumors (22). Esophageal tumors were also observed in the rats treated with NNN, while the NNK-treated rats had significant numbers of lung tumors, not found in the rats treated with NNN. In other studies, NNN administered at only 5 ppm in the drinking water induced esophageal tumors in 71% of the treated rats (23). It is also notable that NNN reproducibly induces tumors of the respiratory tract in both mice and hamsters, and caused malignant nasal tumors in mink (2). The 5'-hydroxylation metabolic activation pathway of NNN has also received relatively limited attention compared to 2'-hydroxylation because studies in rats have associated 2'-hydroxylation with esophageal and nasal carcinogenicity (2). Furthermore, DNA adducts produced by 2'-hydroxylation are the same as those formed by methyl hydroxylation of NNK (Scheme 4), and these have been extensively studied, not only in rats but also in humans (2,9,24-26). But 5'-hydroxylation of NNN predominates in respiratory target tissues (lung and trachea) of mice and rats (2), as well as in hepatic microsomes of humans (27), and in the urine of monkeys treated with NNN (28). Furthermore, adducts resulting from 5'-hydroxylation are unique to NNN, so could be used as specific biomarkers for this carcinogen. It will be important to investigate the relationship of these adducts to NNN carcinogenicity and to determine their presence in human tissues.

In summary, we have characterized dAdo and dThd adducts resulting from the reactions of 5'-acetoxyNNN with deoxyribonucleosides and DNA. These results further our understanding of DNA adduct formation by the quantitatively important 5'-hydroxylation metabolic activation pathway of this carcinogen, encountered daily by more than one billion tobacco users in the world.

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Scheme 1.
Formation of dGuo, dAdo, and dThd adducts from 5'-acetoxyNNN (**3**).

Scheme 2.
Synthesis of adduct **17**.

Scheme 3.
Synthesis of adduct **18**.

Scheme 4.

Pyridyloxobutylation of DNA by methyl hydroxylation of NNK and 2'-hydroxylation of NNN.

Figure 1. Chromatogram obtained upon LC-ESI-MS/MS-SRM analysis, at m/z 399 U m/z 283 (system 5), of the products of reaction of 5'-acetoxyNNN (**3**) and dAdo.

Figure 2.
UV spectrum of peak 1 of Figure 1, identified as 5(S)-**9** (Scheme 1).

Figure 3. Chromatograms obtained upon LC-ESI-MS/MS-SRM analysis, at m/z 401 U m/z 285 (system 5), of the products formed from NaBH_3CN treatment of A) synthetic N^6 -[5-(3-pyridyl)tetrahydrofuran-2-yl]dAdo (**9**) and B) reaction of 5'-acetoxyNNN (**3**) with dAdo.

Figure 4. Chromatograms obtained upon LC-ESI-MS/MS-SRM analysis, at m/z 383 $\rightarrow$ m/z 267 (system 5), of the products formed from NaBH_3CN treatment of A) synthetic 6-[2-(3-pyridyl)pyrrolidin-1-yl]purine-2'-deoxyriboside (**18**) and B) reaction of 5'-acetoxyNNN (**3**) with dAdo.

Figure 5.

Chromatograms obtained upon LC-ESI-MS/MS-SRM analysis, at m/z 392 $\rightarrow$ m/z 276 (system 5), of A) the products formed from NaBH_3CN treatment of the reaction of 5'-acetoxyNNN (3) with dThd and B) co-injection of A with standard O^2 -[4-(3-pyridyl)-4-hydroxybut-1-yl] thymidine (11).

Figure 6.

Chromatograms obtained upon LC-ESI-MS/MS-SRM analysis (system 5) of A) standard adducts **15** (m/z 417 $\rightarrow$ m/z 301), **12** (m/z 399 $\rightarrow$ m/z 283), **17** (m/z 401 $\rightarrow$ m/z 285), **18** (m/z 383 $\rightarrow$ m/z 267), and **11** (m/z 392 $\rightarrow$ m/z 276) of Scheme 1 and B) an NaBH_3CN -treated enzymatic hydrolysate of DNA that had been reacted with 5'-acetoxyNNN and analyzed for these adducts. Each transition used corresponds to $[\text{BH}]^+$. Relative adduct amounts in B, based on peak areas only, were (% of dGuo adduct **15**): **12** (67%); **17** (8%); **18** (7.3%); **11** (0.9%).