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Analysis of Adducts in Hepatic DNA of Rats Treated with *N*-Nitrosopyrrolidine

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Abstract

N-Nitrosopyrrolidine (NPYR) is a hepatocarcinogen in rats. It is metabolically activated by cytochrome P450 enzymes in the liver leading to the formation of 4-oxobutanediazohydroxide (**4**) and related intermediates that react with DNA to form adducts. Since DNA adducts are thought to be critical in carcinogenesis by NPYR, we analyzed hepatic DNA of NPYR-treated rats for several adducts: *N*²-(tetrahydrofuran-1-yl)dGuo (*N*²-THF-dGuo, **13**), *N*⁶-THF-dAdo (**14**), *N*⁴-THF-dCyd (**17**), and dThd adducts **15** and **16**. The rats were treated with NPYR in the drinking water, 600 ppm for 1 week, or 200 ppm for 4 or 13 weeks. Hepatic DNA was isolated, enzymatically hydrolyzed, and analyzed by capillary LC-ESI-MS-SIM, which indicated the presence of adducts **13**, **14**, and **17**. Since these adducts can be unstable at the deoxyribonucleoside level, further analyses were carried out using DNA treated with NaBH₃CN, which converts adducts **13–17** to *N*²-(4-hydroxybut-1-yl)dGuo [*N*²-(4-HOB)-dGuo, **18**], *N*⁶-(4-HOB)-dAdo (**19**), *O*²-(4-HOB)-dThd (**20**), *O*⁴-(4-HOB)-dThd (**21**), and *N*⁴-(4-HOB)-dCyd (**22**). [¹⁵N]-Labeled analogues of adducts **18–20** and **22** were synthesized and used in this analysis, which was performed by capillary LC-ESI-MS/MS-SRM. Convincing evidence for the presence of adducts **18–22** was obtained. Levels of *N*²-(4-HOB)-dGuo (**18**), *N*⁶-(4-HOB)-dAdo (**19**), *O*²-(4-HOB)-dThd (**20**), and *O*⁴-(4-HOB)-dThd (**21**) were (μmol/mol dGuo): 3.41–5.39, 0.02–0.04, 2.56–3.87, and 2.28–5.05, respectively. *N*⁴-(4-HOB)-dCyd (**22**) was not quantified due to interfering peaks. These results provide the first evidence for tetrahydrofuranyl-substituted DNA adducts in the livers of rats treated with NPYR. The finding of dAdo and dThd adducts is of particular interest since previous studies have shown that NPYR causes mutations at AT base pairs in DNA of rat liver.

Keywords

N-nitrosopyrrolidine; DNA adducts

Introduction

N-Nitrosopyrrolidine (NPYR, **1**, Scheme 1) causes liver tumors in rats and respiratory tract tumors in mice and hamsters (1–3). Several lines of evidence indicate that there is significant human exposure to NPYR through the diet, tobacco smoke and endogenous formation (4–8). NPYR requires metabolic activation to exert its mutagenic and presumably its carcinogenic effects (9). The critical step in the metabolic activation of NPYR is cytochrome P450-catalyzed

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α -hydroxylation producing α -hydroxyNPYR (**2**) (10). Compound **2** is unstable and decomposes to produce a cascade of intermediates such as **4**, **7**, **8**, and **12**, which react with DNA to form adducts (11). There is no reason to doubt that DNA adduct formation is critical in carcinogenesis by NPYR. Therefore, we have devoted considerable energy to structural elucidation of NPYR-DNA adducts. Adducts with dGuo have been extensively characterized in previous in vitro studies using α -acetoxyNPYR (**3**) as a precursor to **2** (11). Some of these, such as **5**, **6**, **9**, and **13**, are shown in Scheme 1. In the accompanying paper, we described the first characterization of dAdo, dThd, and dCyd adducts formed by the reactions of α -acetoxyNPYR with DNA (e.g., structures **14–17**, Scheme 1)(12).

Relatively few studies have examined NPYR-DNA adduct formation in vivo. We have identified adducts **5**, **6**, and **9** in hepatic DNA of NPYR-treated rats, and adduct **6** in rat urine (13–16). Shank and co-workers studied the formation and persistence of adduct **6** in liver, lung and kidney of NPYR-treated rats, mice, and hamsters (17,18). There are no previous studies of adducts **13–17** in DNA of NPYR-treated rats. In view of the report by Kanki et al that NPYR causes mutations at A-T base pairs in hepatic DNA of NPYR-treated rats (19), it is particularly important to determine whether adducts with dAdo and dThd are formed in vivo. Therefore, in the present study, we investigated the presence of adducts **13–17** in hepatic DNA of NPYR-treated rats. Since some of these adducts are unstable, we treated the DNA with NaBH₃CN, which converts **13–17** to the stable adducts *N*²-(4-HOB)-dGuo (**18**), *N*⁶-(4-HOB)-dAdo (**19**), *O*²-(4-HOB)-dThd (**20**), *O*⁴-(4-HOB)-dThd (**21**), and *N*⁴-(4-HOB)-dCyd (**22**). [¹⁵N]-Labeled analogues of adducts **18–20** and **22** were synthesized and used in the analysis.

Experimental Procedures

Apparatus and Assay Conditions

HPLC quantitation of dGuo from DNA samples was carried out with an Agilent 1100 Series autosampler (Agilent Technologies, Palo Alto, CA) coupled to a Waters Millipore automated gradient controller and model 6000 A solvent delivery system (Waters Division, Millipore, Milford, MA) equipped with an in-line Gilson Model 116 UV detector (Gilson Medical Electronics, Middleton, WI) and a 4.6 mm $\times$ 25 cm, 5 μ m, Luna C18(2) column (Phenomenex, Torrance, CA) eluted with a gradient from 5 to 45% MeOH in H₂O for 30 min, then from 45 to 100% MeOH over the course of 10 min at a flow rate of 0.7 mL/min with detection at 254 nm.

LC-negative ion-ESI-MS analysis of 4-hydroxybutyraldehyde-2,4-dinitrophenylhydrazone was carried out with a Finnigan LCQ Deca instrument (Thermo Electron, San Jose, CA) interfaced with a Waters Alliance 2690 HPLC multisolvent delivery system and a 4.6 mm $\times$ 25 cm, 5 μ m, Gemini C18 column (Phenomenex) with elution by a gradient from 40 to 70% CH₃CN in H₂O for 40 min at a flow rate 0.5mL/min. The source was set as follows: voltage, 4.2 kV; current, 0.2 μ A; sheath gas pressure, 70 psi; capillary temperature, 350 $^{\circ}$ C; parent mass, 267 amu; isolation width, 1.5 amu; activation amplitude, 30%; activation Q, 0.25; activation time, 30 ms.

Capillary LC-ESI-MS-SIM and LC-ESI-MS/MS-SRM analyses of adducts were carried out with a Finnigan Quantum Ultra AM or a Discovery Max (Thermo Electron, San Jose, CA) triple quadrupole mass spectrometer interfaced with an Agilent 1100 capillary flow HPLC and

a 150 mm × 0.5 mm Zorbax SB C18 column (Agilent) with isocratic elution by 0% CH₃CN in 15 mM ammonium acetate buffer, pH 6.6, for 10 min, then a gradient from 0 to 25% CH₃CN over the course of 29 min, then 25 to 75% CH₃CN for 5 min, then 75% CH₃CN for 5 min, and then returning to 0% CH₃CN in 5 min, at a flow rate of 15 µL/min. The column was operated at 25 °C. The first 10 min of eluant was directed to waste, and the 10–40 min fractions were diverted to the ESI source. The MS parameters were set as follows: spray voltage, 4 kV; sheath gas pressure, 40 psi; capillary temperature, 250 °C; collision energy, 13V; scan width, 0.5 amu; scan time, 0.2 s; Q1 peak width, 0.7 amu; Q3 peak width, 0.7 amu; Q2 collision cell gas pressure, 1.0 mTorr; source CID, 10 V; tube lens offset, 85 V.

Chemicals and Enzymes

[¹⁵N₅]dGuo, [¹⁵N₅]dAdo, [¹⁵N₃]dCyd, and [¹⁵N₂]dThd were obtained from Spectra Stable Isotopes (Columbia, MD). Ethanol was obtained from AAPER Alcohol and Chemical Co. (Shelbyville, KY). 2-Propanol was purchased from Acros Organics (Morris Plains, NJ). Puregene DNA purification solutions were purchased from Gentra Systems (Minneapolis, MN). Strata-X cartridges, 33 µm, 30 mg/1 mL were obtained from Phenomenex. 2,4-Dinitrophenylhydrazine (2,4-DNP) reagent was prepared as described (20). Alkaline phosphatase from calf intestine was obtained from Roche Diagnostics Corporation (Indianapolis, IN). Calf thymus DNA, DNase I, phosphodiesterase I and all other chemicals were purchased from Sigma-Aldrich (St. Louis, MO).

[¹⁵N₅]N²-(4-HOB)-dGuo ([¹⁵N₅]18)

A mixture of [¹⁵N₅]dGuo (1500 µg, 5.5 µmol), 2-ethoxytetrahydrofuran (24 µL), trifluoroacetic acid (0.3 µL), and DMF (150 µL) was allowed to stand for 3 days at room temperature. Then NaBH₃CN (21 mg in 0.87 mL H₂O) was added and the mixture was allowed to stand overnight. The product, [¹⁵N₅]18, was purified by HPLC using two 4.6 mm × 35 cm Luna C18(2) columns eluted with 5% acetonitrile in H₂O for 10 min, then a linear gradient to 30% acetonitrile over 50 min; retention time 46 min. Positive ESI-MS: *m/z* 345 [M + H]⁺, MS/MS of *m/z* 345: *m/z* 229 [BH]⁺.

[¹⁵N₅]N⁶-(4-HOB)-dAdo ([¹⁵N₅]19) and [¹⁵N₃]N⁴-(4-HOB)-dCyd ([¹⁵N₃]22)

These were prepared from [¹⁵N₅]dAdo and [¹⁵N₃]dCyd as described for the unlabelled compounds (12). ¹H-NMR and UV spectra of [¹⁵N₅]19 were the same as those of 19; positive ESI-MS: *m/z* 329 [M + H]⁺, MS/MS of *m/z* 329: *m/z* 213 [BH]⁺, *m/z* 141 [[¹⁵N₅]adenine + H]⁺. The UV spectrum of [¹⁵N₃]22 was the same as that of unlabeled 22; positive ESI-MS: *m/z* 303 [M + H]⁺, MS/MS of *m/z* 303: *m/z* 187 [BH]⁺, *m/z* 115 [[¹⁵N₃]cytosine + H]⁺.

[¹⁵N₂]O²-(4-HOB)-dThd ([¹⁵N₂]20)

[¹⁵N₂]dThd-5'-toluenesulfonate ester (50 mg, 0.125 mmol) was prepared from [¹⁵N₂]dThd as described (21) and converted to [¹⁵N₂]20 (394 µg, 1.25 µmol, 1%) by the method used for the unlabelled compound (12). ¹H-NMR and UV spectra were the same as those of the unlabelled compound; positive ESI-MS: *m/z* 317 [M + H]⁺, MS/MS of *m/z* 317: *m/z* 201 [BH]⁺, *m/z* 129 [[¹⁵N₂]thymine + H]⁺.

Animal Experiments

For Experiment 1, 6 week old male F344 rats were purchased from Charles River (Wilmington, MA) and housed in the Research Animal Resources facility of the University of Minnesota under the following conditions: temperature 20–23 °C; relative humidity 50 ± 10%; 12-h light-dark cycle. They were maintained on NIH-07 diet (Harlan, Madison, WI) and tap water. Two groups, each consisting of 4 rats weighing approximately 250 g each, were given either tap

water or tap water containing 600 ppm NPYR for 1 week, then sacrificed. The same protocol was used previously for detection of NPYR-DNA adducts (14).

For Experiment 2, 6 week old female Sprague-Dawley rats were obtained from Japan SLC (Shizuoka, Japan). They were housed in the Facility of the National Institute of Health Sciences under the following conditions: temperature 23 ± 2 °C; relative humidity $60 \pm 5\%$; ventilation frequency 18 times/h, 12 h light-dark cycle. The rats had free access to normal powdered diet (CRF-1, Oriental Yeast Co., Ltd., Tokyo, Japan) and tap water. Four groups, each consisting of 5 rats weighing approximately 200 g each, were given either tap water or tap water containing 200 ppm NPYR for 4 weeks or 13 weeks, then sacrificed. This protocol was used in the study by Kanki et al (19).

DNA isolation and analysis

Hepatic DNA was isolated as described previously, except that NaBH_3CN was not added to homogenized tissues and solutions used in the procedure (22).

For analysis of adducts **13–17**, an aliquot of the DNA was hydrolyzed enzymatically, as described (23), and another aliquot was treated by neutral thermal hydrolysis (100 °C, pH 7.0, 1h) (24), then analyzed for the above adducts.

For analysis of 4-hydroxybutyraldehyde-2,4-dinitrophenylhydrazone, a 0.8 mL aliquot of the neutral thermal hydrolysate was allowed to react with 20 μL of 0.5 $\mu\text{g}/\mu\text{L}$ 2,4-DNP reagent. The mixture was extracted with CH_2Cl_2 , the extract concentrated, and analyzed by LC-negative ion-ESI-MS-SIM at m/z 267.

For analysis of adducts **18–22**, 1–2.5 mg DNA was treated with NaBH_3CN as described (23). Briefly, the DNA was dissolved in 1.0 mL of 10 mM Tris-HCl/5mM MgCl_2 buffer, pH 7.0, containing DNase I (1300 units). NaBH_3CN (10 mg) was added to the mixture three times. After the first 2 additions, the mixture was allowed to stand at room temperature for 30 min. After the final addition, it was allowed to stand at 37 °C for 30 min. The pH was adjusted to 7 with HCl and the internal standards $[\text{N}^{15}_5]\text{N}^2\text{-(4-HOB)-dGuo}$ (1956 fmol), $[\text{N}^{15}_5]\text{N}^6\text{-(4-HOB)-dAdo}$ (23 fmol), $[\text{N}^{15}_2]\text{O}^2\text{-(4-HOB)-dThd}$ (468 fmol) and $[\text{N}^{15}_3]\text{N}^4\text{-(4-HOB)-dCyd}$ (765 fmol) - quantified by NMR with toluene as standard were added. The resulting mixture was incubated with an additional amount of DNase I (1300 units) at 37 °C for 10 min, then phosphodiesterase I (0.06 units) and alkaline phosphatase (380 units) were added, and the incubation was continued for another 60 min. A 10 μL aliquot was removed for dGuo analysis, and the remainder was applied to a Strata-X cartridge. The cartridge was sequentially washed with 1.0 mL of H_2O , 2.0 mL of 20% MeOH in H_2O , and 2 mL 100% MeOH. The MeOH-containing eluants were combined, evaporated to dryness, and redissolved in 200 μL of MeOH for transfer into an autosampler vial with an infused 300 μL insert (Chrom Tech), then dried again under vacuum. The residue was redissolved in 20 μL of H_2O , and 8 μL was analyzed by LC-ESI-MS/MS-SRM.

Calibration curves were constructed from varying amounts of the adduct standards and a fixed amount of the $[\text{N}^{15}]$ -labeled standard as follows: $\text{N}^2\text{-(4-HOB)-dGuo}$ (**18**) (10 – 100 fmol), $[\text{N}^{15}_5]\text{N}^2\text{-(4-HOB)-dGuo}$ (260 fmol); $\text{N}^6\text{-(4-HOB)-dAdo}$ (**19**) (3 – 150 fmol); $[\text{N}^{15}_5]\text{N}^6\text{-(4-HOB)-dAdo}$ (75 fmol); $\text{O}^2\text{-(4-HOB)-dThd}$ (1 – 67 fmol) (**20**); $[\text{N}^{15}_2]\text{O}^2\text{-(4-HOB)-dThd}$ (31 fmol). The amounts of standards used for the calibration curves were based on the expected amounts of adducts. Detection limits were 0.5–2 fmol adduct, starting with 0.5–1 mg of DNA.

Results

We prepared [^{15}N]-labeled internal standards to assist in adduct identification and quantitation. [$^{15}\text{N}_5$] N^2 -(4-HOB)-dGuo ([$^{15}\text{N}_5$]**18**) was prepared by reaction of 2-ethoxytetrahydrofuran with [$^{15}\text{N}_5$]dGuo in the presence of trifluoroacetic acid, followed by reduction with NaBH_3CN . [$^{15}\text{N}_5$] N^6 -(4-HOB)-dAdo ([$^{15}\text{N}_5$]**19**) and [$^{15}\text{N}_3$] N^4 -(4-HOB)-dCyd ([$^{15}\text{N}_3$]**22**) were prepared by NaBH_3CN reduction of [$^{15}\text{N}_5$] N^6 -THF-dAdo and [$^{15}\text{N}_3$] N^4 -THF-dCyd, which were isolated from reactions of α -acetoxyNPYR with [$^{15}\text{N}_5$]dAdo and [$^{15}\text{N}_3$]dCyd, respectively. [$^{15}\text{N}_2$] O^2 -(4-HOB)-dThd ([$^{15}\text{N}_2$]**20**) was prepared by the reaction of [$^{15}\text{N}_2$]dThd-5'-tosylate with 1,4-butanediol as described for the unlabeled adduct (**12**).

Calibration curves relating amounts and MS peak areas, expressed as ratios of N^2 -(4-HOB)-dGuo (**18**), N^6 -(4-HOB)-dAdo (**19**), and O^2 -(4-HOB)-dThd (**20**) to the corresponding internal standards, were constructed for quantitation of adduct levels in the DNA samples. All curves were linear ($R^2 = 0.99$) in the range measured. A calibration curve for [$^{15}\text{N}_3$] N^4 -(4-HOB)-dCyd ([$^{15}\text{N}_3$]**22**) was not constructed because adduct **22** was not quantified (see below).

Two experiments were performed. In Experiment 1, male F344 rats were treated with NPYR in the drinking water (600 ppm) for 1 week. In Experiment 2, female Sprague-Dawley rats were treated with NPYR in the drinking water (200 ppm) for 4 or 13 weeks.

LC-ESI-MS-SIM chromatograms of an enzyme hydrolysate of DNA isolated from the liver of an NPYR-treated F-344 rat are illustrated in Figure 1. Figure 1A-D show SIM at m/z 338 [$(\text{M} + \text{H})^+$ of N^2 -THF-dGuo(**13**)], m/z 322 [N^6 -THF-dAdo(**14**)], m/z 313 [dThd adducts **15** and **16**] and m/z 298 [N^4 -THF-dCyd(**17**)]. The indicated peaks had the correct retention times for these adducts and disappeared upon neutral thermal hydrolysis (Figure 1E-H), consistent with their known properties (**12**). The dThd adducts were not observed in this analysis, presumably due to their instability resulting in decomposition during DNA isolation and enzyme hydrolysis.

The neutral thermal hydrolysate obtained in this experiment was treated with 2,4-DNP reagent. Analysis by LC-negative ion-ESI-MS-SIM at m/z 267 [$(\text{M} - \text{H})^-$ for 4-hydroxybutyraldehyde-2,4-dinitrophenylhydrazone] produced the chromatogram illustrated in Figure 2B. The MS/MS data shown in Figure 2 C, D are characteristic of 2,4-dinitrophenylhydrazones (**25**) and confirmed the structure, demonstrating that neutral thermal hydrolysis of this DNA produced 2-hydroxytetrahydrofuran (**11**), the cyclic and predominant form of 4-hydroxybutyraldehyde (**10**).

Treatment of the DNA with NaBH_3CN , which converts the adducts to the stable 4-HOB derivatives N^2 -(4-HOB)-dGuo (**18**), N^6 -(4-HOB)-dAdo (**19**), O^2 -(4-HOB)-dThd (**20**), O^4 -(4-HOB)-dThd (**21**), and N^4 -(4-HOB)-dCyd (**22**), was used for analysis. LC-ESI-MS/MS-SRM chromatograms of an enzyme hydrolysate of NaBH_3CN -treated DNA isolated from the liver of an F-344 rat treated with NPYR (Experiment 1) are illustrated in Figure 3. SRM was carried out at m/z 340 $\rightarrow$ m/z 224 for N^2 -(4-HOB)-dGuo (**18**), m/z 324 $\rightarrow$ m/z 208 for N^6 -(4-HOB)-dAdo (**19**), m/z 315 $\rightarrow$ m/z 199 for O^2 -(4-HOB)-dThd (**20**) and O^4 -(4-HOB)-dThd (**21**), and m/z 300 $\rightarrow$ m/z 184 for N^4 -(4-HOB)-dCyd (**22**). The chromatograms provide clear evidence for the presence of each adduct, having peaks co-eluting with the appropriate [^{15}N]-labeled internal standards. (No [^{15}N]-labelled internal standard was available for O^4 -(4-HOB)-dThd but it had the correct retention time, based on comparison to the unlabelled standard **21**, and was quantified using the calibration curve for **20**). Similar chromatograms were obtained upon analysis of the Sprague-Dawley rat liver DNA samples (Experiment 2). Peaks corresponding to the retention times of N^2 -(4-HOB)-dGuo, O^2 -(4-HOB)-dThd and O^4 -(4-HOB)-dThd were not observed in hydrolysates of hepatic DNA from control rats. Small peaks having the same retention times as N^6 -(4-HOB)-dAdo and N^4 -(4-HOB)-dCyd were observed in some control

liver samples. Because of these peaks, levels of N^6 -(4-HOB)-dAdo could only be estimated and N^4 -(4-HOB)-dCyd could not be quantified.

Levels of the adducts are summarized in Table 1. N^2 -(4-HOB)-dGuo, O^2 -(4-HOB)-dThd and O^4 -(4-HOB)-dThd were formed in comparable amounts which were higher than those of N^6 -(4-HOB)-dAdo. Adduct levels were similar in the rats from Experiments 1 and 2.

The stability of the adducts in DNA at 37 °C was investigated to determine whether they might be spontaneously lost in the rat. Hepatic DNA from an NPYR treated rat (Experiment 2) was kept at 37 °C overnight, then treated with NaBH₃CN and analyzed as above. There was no significant decomposition of the adducts summarized in Table 1, suggesting that they are chemically stable in rat liver.

Discussion

The results of this study clearly demonstrate that quantifiable levels of N^2 -THF-dGuo (**13**) and dThd adducts **15** and **16** are present in hepatic DNA of NPYR-treated rats. We also obtained convincing evidence for the presence of N^6 -THF-dAdo (**14**) and N^4 -THF-dCyd (**17**) but quantitation was more difficult due to their low amounts and the presence of interfering peaks in some samples. Several lines of evidence support these conclusions. First, peaks corresponding in retention time to N^2 -THF-dGuo (**13**), N^6 -THF-dAdo (**14**), and N^4 -THF-dCyd (**17**) were observed in the SIM chromatograms (Figure 1A, B, D) and these disappeared upon neutral thermal hydrolysis, consistent with the properties of these adducts (12). Second, treatment of the neutral thermal hydrolysates of the hepatic DNA with 2,4-DNP reagent confirmed the expected release of 2-hydroxytetrahydrofuran (**11**) from the adducts (Figure 2). Third, treatment of the DNA with NaBH₃CN converted the adducts to N^2 -(4-HOB)-dGuo (**18**), N^6 -(4-HOB)-dAdo (**19**), O^2 -(4-HOB)-dThd (**20**), O^4 -(4-HOB)-dThd (**21**), and N^4 -(4-HOB)-dCyd (**22**) which were identified by comparison of their retention times to those of [¹⁵N]-labeled internal standards in the SRM chromatograms (Figure 3). These results demonstrate that the in vivo α -hydroxylation pathway of NPYR metabolic activation to hepatic DNA adducts proceeds by way of intermediates **4** and **7** to oxonium ion **12**, or a related cyclic intermediate.

Our results indicate that adducts **13–16** are chemically stable in DNA at 37 °C. The adduct levels observed in our study (Table 1) therefore probably represent a steady state between adduct formation and repair under conditions of chronic NPYR treatment, but this requires further study. While dThd adducts **15** and **16** are stable in DNA, they are quite unstable at the deoxyribonucleoside level (12), which prevents their quantitation in the absence of NaBH₃CN treatment of the DNA.

In the study by Kanki et al, Sprague-Dawley *gpt* delta rats were given NPYR in the drinking water at a concentration of 200 ppm for 13 weeks, as in Experiment 2 (19). At this dose, histopathologically altered, mostly clear, hepatocellular foci were frequently observed in the NPYR-treated rats. GST-P-positive liver cell foci corresponding to these foci were significantly increased. Treatment with NPYR led to a 10 fold increase in *gpt* mutant frequency compared to controls. The predominant type of base substitution, and the only one more frequently observed than in controls, was an A:T to G:C transition which was seen in 33 (49.3%) of the 67 mutations reported. This mutation was not observed in control rats. The presence of dAdo and dThd adducts **14–16** in the hepatic DNA of the rats in our study provide a mechanistic explanation for the mutations observed in the study by Kanki et al. These results are particularly notable because previous studies have only characterized dGuo adducts in DNA samples from rodents treated with NPYR. Furthermore, the levels of the dThd adducts in aggregate are comparable to those of the dGuo adducts measured here (Table 1). Collectively,

these studies suggest that dThd adducts may be very important in hepatocarcinogenesis by NPYR. In vitro mutagenesis studies are required to assess the miscoding potential of adducts **14–16**.

A limitation of this study is the method of quantitation of N^2 -THF-dGuo (**13**), N^6 -THF-dAdo (**14**), and dThd adducts **15** and **16**. While NaBH_3CN treatment of DNA converts these adducts to the corresponding stable 4-(HOB)-adducts **18–21**, the efficiency of this conversion is uncertain. We did not have DNA containing [^{15}N]-labeled **13–16** available for use as an internal standard. Therefore, the data in Table 1 may underestimate the levels of adducts **13–16**.

Several previous studies of reactants other than α -acetoxyNPYR have demonstrated the in vitro formation of adducts resulting from the reaction of oxonium ions, lactols, or related intermediates with deoxyribonucleosides or DNA. Structures of these adducts are illustrated in Figure 4. In all cases, reaction occurs via the exocyclic amino group of dGuo. Adduct **23** is formed by the reaction of α -acetoxy-*N*-nitrosopiperidine or 2-hydroxytetrahydro-2*H*-pyran with dGuo or DNA (24,26). Adduct **24** has been observed in reactions of 4-(acetoxymethylnitrosamino)-1-(3-pyridyl)-1-butanone with dGuo (27). Adduct **25** is a product of the reaction of acetaldehyde or aldoxane with dGuo and DNA (23,28), while adduct **26** is similarly produced from crotonaldehyde and paralldol (28,29). Adduct **27** is a product of the reaction of glucose with dGuo (30,31). The results presented here are, to our knowledge, the first demonstration of the in vivo formation of adducts of this structural type.

In summary, the results of this study demonstrate the presence of adducts **13–17** in hepatic DNA of NPYR treated rats. These types of adducts are derived from cyclic oxonium ions or related intermediates and have not been previously reported in vivo. The dAdo and dThd adducts **14–16** may be particularly important in NPYR carcinogenesis because a previous study demonstrates that mutations at A:T base pairs are common in hepatic DNA of NPYR-treated rats.

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Abbreviations

NPYR	<i>N</i> -nitrosopyrrolidine
THF	tetrahydrofuran-2-yl
4-HOB	4-hydroxybut-1-yl
2	4-DNP, 2,4-dinitrophenylhydrazine

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Figure 1.

Chromatograms obtained upon LC-ESI-MS-SIM analysis of an enzyme hydrolysate of hepatic DNA of an F-344 rat treated with NPYR in the drinking water (Experiment 1, 600 ppm for 1 week). A-D, SIM for: A) m/z 338 $[[M + H]^+]$ of N^2 -THF-dGuo (**13**); B) m/z 322 $[[M + H]^+]$ of N^6 -THF-dAdo(**14**); C) m/z 313 $[[M + H]^+]$ of dThd adducts **15** and **16**; and D) m/z 298 $[[M + H]^+]$ of N^4 -THF-dCyd(**17**). The indicated peaks had the same retention times as standards. No peaks were observed in panel C due to the instability of the dThd adducts. Under these HPLC conditions, two diastereomers of N^2 -THF-dGuo (**13**) are separated and 3 dAdo peaks are observed, the first 2 corresponding in retention time to 2 diastereomers of N^6 -THF-dAdo (**14**), and the third being of unknown structure (**12**). E-H, analysis for the same adducts as in A-D, except after neutral thermal hydrolysis. The y-axes of E-H are the same as those of A-D in terms of absolute ion signals. All adduct peaks observed in A-D disappeared.

Figure 2. Chromatograms obtained upon LC-negative ion-ESI-MS-SIM (m/z 267) of A) standard 4-hydroxybutyraldehyde-2,4-dinitrophenylhydrazone and B) neutral thermal hydrolysate of hepatic DNA from an NPYR-treated rat (Experiment 1). Panels C and D are the MS/MS spectra of m/z 267 of the standard and the 20.8 min peak from panel B, respectively.

Figure 3.

Chromatograms obtained upon LC-ESI-MS/MS-SRM analysis of an enzyme hydrolysate of $NaBH_3CN$ -treated hepatic DNA of an F-344 rat administered NPYR in the drinking water (Experiment 1, 600 ppm for 1 week). A) SRM for m/z 340 $\rightarrow$ m/z 224 for N^2 -(4-HOB)-dGuo (**18**) and m/z 345 $\rightarrow$ m/z 229 for $[^{15}N_5]$ **18**; B) m/z 324 $\rightarrow$ m/z 208 for N^6 -(4-HOB)-dAdo (**19**) and m/z 329 $\rightarrow$ m/z 213 for $[^{15}N_5]$ **19**; C), m/z 315 $\rightarrow$ m/z 199 for O^2 -(4-HOB)-dThd (**20**) and O^4 -(4-HOB)-dThd (**21**) and m/z 317 $\rightarrow$ m/z 201 for $[^{15}N_2]$ **20**; and D) m/z 300 $\rightarrow$ m/z 184 for N^4 -(4-HOB)-dCyd (**22**) and m/z 303 $\rightarrow$ m/z 187 $[^{15}N_3]$ **22**.

Figure 4.
Structures of N^2 -dGuo lactol type adducts reported in the literature.

Scheme 1. Overview of Adduct Formation from NPYR (1) and α -AcetoxyNPYR (3)

Table 1
Levels of adducts in NaBH₃CN-treated hepatic DNA of rats given NPYR in the drinking water.

	$\mu\text{mol/mol dGuo}^a$			
	<i>N</i> ² -(4-HOB)-dGuo (18)	<i>N</i> ⁶ -(4-HOB)-dAdo (19)	<i>O</i> ² -(4-HOB)-dThd (20)	<i>O</i> ⁴ -(4-HOB)-dThd (21)
Male F-344 600 ppm, 1 week	3.41 ± 0.60	0.02 ± 0.01	2.56 ± 0.84	2.28 ± 0.47
Female Sprague Dawley 200 ppm, 4 weeks	4.84 ± 0.48	0.03 ± 0.01	3.87 ± 0.97	3.83 ± 0.57
200 ppm, 13 weeks	5.39 ± 0.39	0.04 ± 0.02	3.52 ± 0.43	5.05 ± 1.68

^aDNA was isolated from liver, treated with NaBH₃CN, enzymatically hydrolyzed, and analyzed by LC-ESI-MS/MS-SRM using [¹⁵N]-labeled adducts **18–20** (added at the beginning of the enzyme hydrolysis) as internal standards. Values are mean ± S.D. of single analyses of hydrolysates from 3–4 rats per group.