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## Formation and Accumulation of Pyridyloxobutyl DNA Adducts in F344 Rats Chronically Treated With 4-(Methylnitrosamino)-1-(3-pyridyl)-1-butanone and Enantiomers of Its Metabolite, 4-(Methylnitrosamino)-1-(3-pyridyl)-1-butanol

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### Abstract

4-(Methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK, **1**) and its metabolite, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL, **2**) are both potent pulmonary carcinogens in rats. The metabolism of NNK to NNAL is stereoselective and reversible, with (*S*)-NNAL being the major enantiomer formed from NNK. In rats, (*R*)-NNAL undergoes facile glucuronidation and is rapidly excreted in urine, while (*S*)-NNAL is preferentially retained in tissues and converted to NNK. We hypothesized that the lung carcinogenicity of NNK in the rat is due in part to preferential retention of (*S*)-NNAL in lung, reversion to NNK, then metabolic activation of NNK to pyridyloxobutyl (POB)-DNA adducts. We tested this hypothesis by treating male F344 rats with 10 ppm of NNK, (*R*)-NNAL, or (*S*)-NNAL in the drinking water. After 1, 2, 5, 10, 16, or 20 weeks of treatment, POB-DNA adducts in liver and lung DNA were quantified by HPLC-ESI-MS/MS. At each time point, total adduct levels were higher in lung than liver. *O*<sup>2</sup>-[4-(3-pyridyl)-4-oxobut-1-yl]thymidine (*O*<sup>2</sup>-POB-dThd, **13**) was the major adduct detected. Total adduct levels in the rats treated with (*S*)-NNAL were 0.6–1.3 times as great as those in the NNK group in lung and 0.7–1.4 times in liver, and 6–14 times higher than those in the (*R*)-NNAL group in lung and 11–17 times in liver. These results suggest that (*S*)-NNAL is stereoselectively retained in tissues. This study demonstrates for the first time the accumulation and persistence of specific POB-DNA adducts in rat lung and liver during chronic treatment with NNK, (*R*)-NNAL and (*S*)-NNAL, and supports the hypothesis that preferential retention of (*S*)-NNAL in the lung, followed by reversion to NNK, then metabolic activation of NNK, is critical for lung carcinogenesis by NNK and NNAL.

### Introduction

4-(Methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK, **1**, Chart 1), a tobacco-specific nitrosamine found only in tobacco products and their smoke, is a well-established pulmonary carcinogen in rodents, inducing tumors independent of the route of administration (1). Its lung tumorigenicity is particularly great in the rat, in which it also induces tumors of the liver, pancreas, and nasal cavity (2). NNK and the related tobacco-specific nitrosamine, *N'*-nitrosornicotine (NNN), are considered by the International Agency for Research on Cancer to be human carcinogens (3).

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NNK requires metabolism to express its mutagenicity and presumably its carcinogenicity (1). In this study, we investigate the hypothesis that metabolism of NNK to (*S*)-4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol [(*S*)-NNAL, (*S*)-**2**], followed by reoxidation to NNK, is an important step leading to pulmonary DNA adduct formation in the rat. DNA adducts can cause permanent mutations and are critical in cancer induction by genotoxic carcinogens such as NNK (4). Cytochrome P450-catalyzed  $\alpha$ -hydroxylation is the major bioactivation pathway by which NNK forms DNA adducts and expresses its carcinogenicity.  $\alpha$ -Methyl hydroxylation of NNK (Scheme 1) results in the formation of a reactive electrophile **9**, which pyridyloxobutylates DNA to produce pyridyloxobutyl (POB)-DNA adducts. The formation of POB-DNA adducts is believed to be an important mechanism of NNK carcinogenesis in rodents (5–9), and likely in smokers (10–11). The structures of four POB-DNA adducts -  $O^6$ -[4-(3-pyridyl)-4-oxobut-1-yl]-2'-deoxyguanosine ( $O^6$ -POB-dGuo, **12**),  $O^2$ -[4-(3-pyridyl)-4-oxobut-1-yl]thymidine ( $O^2$ -POB-dThd, **13**), 7-[4-(3-pyridyl)-4-oxobut-1-yl]-2'-deoxyguanosine (7-POB-dGuo, **14**), and  $O^2$ -[4-(3-pyridyl)-4-oxobut-1-yl]-2'-deoxycytidine ( $O^2$ -POB-dCyd, **15**) - have been characterized (Chart 2) (12–14). A sensitive high performance liquid chromatography (HPLC)-electrospray ionization (ESI)-tandem mass spectrometry (MS/MS) method has been developed for quantitative analysis of four stable POB-DNA adducts:  $O^6$ -POB-dGuo,  $O^2$ -POB-dThd, 7-[4-(3-pyridyl)-4-oxobut-1-yl]guanine (7-POB-Gua, **16**) and  $O^2$ -[4-(3-pyridyl)-4-oxobut-1-yl]cytosine ( $O^2$ -POB-Cyt, **17**) (Chart 2) (15).  $\alpha$ -Hydroxylation of NNK at the methylene carbon adjacent to the *N*-nitroso group generates methanediazohydroxide (**10**), which methylates DNA producing methyl-DNA adducts including  $O^6$ -methylGua (**18**), 7-methylGua (**19**) and  $O^4$ -methylThy (**20**) (Scheme 1) (1).

A common pathway of NNK metabolism in virtually all biological systems examined is conversion to NNAL, catalyzed by carbonyl reductases, 11 $\beta$ -hydroxysteroid dehydrogenase type 1 (11 $\beta$ -HSD1), and the aldo-keto reductases AKR1C1–4 (16). NNAL, like NNK, is a potent pulmonary carcinogen in rats (1). NNAL can also be bioactivated through cytochrome P450-catalyzed  $\alpha$ -hydroxylation to form both methyl-DNA adducts (**18–20**) and pyridylhydroxybutyl (PHB)-DNA adducts (Scheme 1) (1,14,17).

Carbonyl reduction of NNK to NNAL is stereoselective and reversible. Several lines of evidence indicate that (*S*)-NNAL is important in lung tumorigenesis by NNK. (*S*)-NNAL is the predominant enantiomer formed from NNK in liver and lung microsomes and cytosol (16,18). Both (*S*)-NNAL and (*R*)-NNAL can be enzymatically reoxidized to NNK, but the reductive pathway predominates (18–21). In rat liver microsomes, reoxidation of (*S*)-NNAL to NNK was significantly faster than of (*R*)-NNAL (18). In experiments carried out *in vivo* in rats, (*S*)-NNAL was stereoselectively retained in lung and was more extensively reconverted to NNK than was (*R*)-NNAL, which was excreted extensively as glucuronides (20,21). Twenty-four hours after administration of racemic [5-<sup>3</sup>H]NNAL to rats, NNAL comprised an average of 75% of total radioactivity in the lung with an (*S*):(*R*) ratio of >20 (20). When [5-<sup>3</sup>H]NNAL enantiomers were administered to rats, less than 5% of the (*R*)-NNAL dose was excreted as NNK or NNK metabolites, compared to almost 20% of (*S*)-NNAL. In A/J mice, (*S*)-NNAL, with activity similar to NNK, was significantly more potent than (*R*)-NNAL or racemic NNAL (19). Finally, the ratios of (*S*):(*R*)-NNAL and their glucuronides increased over time in the urine of humans who stopped using tobacco products, indicating the preferential retention of (*S*)-NNAL (22).

Based on these and related data, we hypothesize that the following sequence of events is important for lung tumorigenesis by NNK: metabolism to (*S*)-NNAL; preferential retention of (*S*)-NNAL in the lung; reoxidation to NNK; and metabolic activation of NNK to POB-DNA adducts (Scheme 2). In this study, we further explored this mechanism by investigating the formation of POB-DNA adducts in rats treated with NNK, (*R*)-NNAL and (*S*)-NNAL. The formation of POB-DNA adducts is an indication of the conversion of NNAL to NNK, since

they can be formed from NNK, but not NNAL. If our hypothesis were correct, then similar POB-DNA adduct levels would be expected in rats treated with (*S*)-NNAL and NNK, while lower levels of adducts would be expected upon (*R*)-NNAL treatment. DNA pyridyloxobutylation of hepatic DNA in rats treated with a single dose of racemic NNAL was previously demonstrated by analysis of 4-hydroxy-1-(3-pyridyl)-1-butanone (HPB, **8**)-releasing DNA adducts by gas chromatography-mass spectrometry (23). In this study, F344 rats were given 10 ppm of NNK, (*R*)-NNAL or (*S*)-NNAL in the drinking water for 20 weeks, and the amounts of individual POB-DNA adducts were quantified in liver and lung DNA by HPLC-ESI-MS/MS analysis.

## Materials and methods

### Caution

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

### Chemicals

NNK was purchased from Toronto Research Chemicals (Toronto, Canada). Purity was greater than 98%. (*R*)- and (*S*)-NNAL were synthesized, as described previously (24). Chemical purities were at least 99% based on normal phase HPLC analysis (24). Enantiomeric purities were greater than 99% by chiral stationary phase -gas chromatography- nitrosamine selective detection analysis (25). POB-DNA adducts were synthesized (15,26). Micrococcal nuclease (LS004797, 15 kU) and phosphodiesterase II (LS003603, 10 U) were obtained from Worthington Biochemical Corporation (Lakewood, NJ). Alkaline phosphatase (567752, 30 U/ $\mu$ L) was purchased from Roche Molecular Biochemicals (Indianapolis, IN). The reagents and enzymes for DNA isolation were purchased from Gentra Systems (Minneapolis, MN). All other chemicals and solvents were acquired from Sigma-Aldrich Chemical Co. (Milwaukee, WI) or Fisher Scientific (Fairlawn, NJ).

### Animal experiment

The study was approved by the University of Minnesota Institutional Animal Care and Use Committee. A total of 216 male F344 rats, 7 weeks old, were purchased from Charles River Laboratories (Kingston, NY). They were housed two animals per micro-isolator cage with corn cob bedding in the Research Animal Resources facility of the University of Minnesota under the following conditions: temperature 20–24 °C; relative humidity 50  $\pm$  10%; 12 h light/dark cycle. They were given NIH-07 diet (Harlan, Madison, WI) and tap water *ad libitum*. The animals were allowed one week for acclimatization to the facility prior to carcinogen treatment. The rats were randomly divided into four groups of 54 rats: (1) control; (2) NNK; (3) (*R*)-NNAL; (4) (*S*)-NNAL. Mean body weights at the beginning of the experiment were: 170  $\pm$  12 g for control group; 174  $\pm$  8 g for NNK group; 181  $\pm$  7 g for (*R*)-NNAL group; 179  $\pm$  11 g for (*S*)-NNAL group. Body weights were recorded once a month. The rats in the treatment groups received 10 ppm of the appropriate carcinogen in the drinking water, and the control rats were given tap water. Aqueous solutions of carcinogens were prepared weekly and stored at 4°C, conditions under which they are known to be stable. These solutions were placed in the plastic water bottles of the rat cages twice weekly and water consumption was recorded. Nine rats per group were sacrificed by CO<sub>2</sub> overdose at 1, 2, 5, 10, 16, and 20 weeks. Tissues were harvested and stored at –80°C until DNA isolation.

### Quantitation of POB-DNA adducts by HPLC-ESI-MS/MS-SRM

DNA was isolated from liver and lung of three rats per group, by following the modified Puregene® DNA isolation protocol (Gentra Systems), as previously described except that

NaBH<sub>3</sub>CN was not used (27). DNA was analyzed for POB-DNA adducts by HPLC-ESI-MS/MS-selected reaction monitoring (SRM), as described previously (15). The characteristics of the method including limits of detection, accuracy, precision, and recoveries have been presented (15). In brief, 0.5–2 mg of each DNA sample, with addition of four deuterated internal standards, was subjected to neutral thermal hydrolysis (100 °C, 30 min), followed by enzymatic hydrolysis with micrococcal nuclease, phosphodiesterase II and alkaline phosphatase. The hydrolysate was partially purified by solid phase extraction (Strata-X cartridge, Phenomenex, CA). Then the sample was delivered by an Agilent 1100 capillary HPLC system (Agilent Technologies, Inc. Wilmington, DE) into a Finnigan TSQ Quantum Discovery Max triple quadrupole mass spectrometer (Thermo Electron, San Jose, CA). The ESI source was operated in the positive mode. POB-DNA adducts and corresponding internal standards were detected by monitoring the formation of the POB ion from the parent ion. The ion transitions are listed in Table 1. Contents of dGuo, dCyd and dThd were determined by HPLC (15). The amount of DNA was calculated from dGuo content by considering that 1 mg of DNA contains 3 μmol nucleotides (28), while dGuo accounts for 22% of total nucleotides in rat DNA, as previously determined in our laboratory. Adduct levels were expressed as fmol adduct/mg DNA. Adduct levels at the 20-week time point were also expressed as fmol per μmol appropriate nucleoside, i.e. fmol *O*<sup>2</sup>-POB-dThd/μmol dThd and fmol *O*<sup>2</sup>-POB-Cyt/μmol dCyd, as listed in Table 3 in the Supporting Information. Levels of POB-DNA adducts were expressed as the mean values ± S.D. of single analyses of three DNA samples per group.

### Statistical analyses

The method of analysis for water consumption was a repeated measures approach using a generalized linear mixed model, which includes both fixed and random effects. The groups and time intervals are considered fixed effects and the rate of change over time for each cage is treated as a random effect. The variance/covariance structure for the data was estimated using restricted maximum likelihood (REML). Two-way analysis of variance (ANOVA) was used for comparisons of levels of total and individual POB-DNA adducts between groups (NNK, (*R*)-NNAL and (*S*)-NNAL) and time (1, 2, 5, 10, 16, 20 weeks). Since the individual adducts were obtained from the same tissue sample, a repeated measures ANOVA was chosen to compare these POB-DNA adducts within each group. Time was treated as a fixed effect and, again, REML was used to estimate the variance/covariance structure. Due to a highly skewed distribution, the POB-DNA adducts were analyzed in the natural log scale. Adjustments for multiple comparisons between groups or between POB-DNA adducts were made according to the Tukey method. A *p*-value < 0.05 was considered to be statistically significant, except for comparisons of individual POB-DNA adducts within each group at each time point, for which a more restrictive *p*-value cutoff of less than 0.01 was used to judge statistical significance.

## Results

### Animal Experiment

The design was based on a carcinogenicity study of NNK and NNAL, in which male F344 rats were given 5 ppm of NNK or NNAL in the drinking water for 128 weeks (2). Twenty-seven of 30 NNK-treated rats and 26 of 30 NNAL-treated rats had lung tumors compared to 6/80 controls. In a preliminary study for this project, POB-DNA adducts were readily detected in liver DNA isolated from rats treated with 2 ppm of NNK in the drinking water for 8 weeks (data not shown). Another study demonstrated that less than 5% of (*R*)-NNAL and about 20% of (*S*)-NNAL was converted to NNK in rats (21). To ensure the detection of POB-DNA adducts at each time point in rats treated with the enantiomers of NNAL, the 10 ppm doses of (*R*)-NNAL and (*S*)-NNAL were chosen for the current experiment. The same dose of NNK was used for comparison.

Overall, the rats treated with (*R*)-NNAL consumed more water than those in the control group, the NNK group and the (*S*)-NNAL group. The differences were significant ( $P < 0.05$ ). There was a small but significant decrease in water consumption over time in each group ( $P < 0.05$ ) (Figure 1A-D). There were no effects of carcinogen treatment on body weights (Figure 1E). All rats survived the 20-week experiment.

### POB-DNA adducts in NNK-treated rats

POB-DNA adducts were not detected in liver and lung DNA isolated from control rats, but were readily detected in liver and lung DNA from NNK-treated rats (Figure 2A–D). The amounts of POB-DNA adducts in NNK-treated rats are summarized in Figure 3A–C (also see Tables 1A and 2A in the Supporting Information). After the first week of treatment, levels of  $O^2$ -POB-dThd were the highest among four POB-DNA adducts in both liver and lung DNA: 650 fmol/mg DNA in liver and 1080 fmol/mg DNA in lung. Those of 7-POB-Gua were 490 fmol/mg DNA in liver and 750 fmol/mg DNA in lung, while those of  $O^2$ -POB-Cyt were 170 fmol/mg DNA in liver and 240 fmol/mg DNA in lung.  $O^6$ -POB-dGuo was not detected in liver DNA, and its level in lung DNA was 45 fmol/mg DNA. At each sacrifice interval, the relative adduct levels in lung and liver DNA were in the same order:  $O^2$ -POB-dThd > 7-POB-Gua >  $O^2$ -POB-Cyt >  $O^6$ -POB-dGuo (Figure 3A–C). In liver, there was a significant difference between levels of each individual adduct ( $P < 0.01$ ), except for levels of 7-POB-Gua and  $O^2$ -POB-dThd at week one, and levels of 7-POB-Gua and  $O^2$ -POB-Cyt at week 20. In lung, levels of individual adducts were significantly different from each other ( $P < 0.01$ ), except for 7-POB-Gua and  $O^2$ -POB-dThd at weeks 16 and 20. Statistical comparisons between each POB-DNA adduct within the NNK group are shown in Table 4A in the Supporting Information.  $O^2$ -POB-dThd was the major adduct detected. It accounted for 49.6–70.0% of total adducts in liver DNA and 51.1–73.4% in lung DNA. The percentage of  $O^2$ -POB-dThd of the adducts measured increased throughout the course of NNK treatment (Tables 1A and 2A in the Supporting Information). The percentage of 7-POB-Gua of total POB adduct levels measured decreased over time, accounting for 18.7–39.1% in liver DNA and 15.0–35.5% in lung DNA. Those of  $O^2$ -POB-Cyt were 6.2–13.0% in liver DNA and 6.5–13.3% in lung DNA.  $O^6$ -POB-dGuo contributed to 0.3–2.1% of total adduct levels in lung DNA. The levels of  $O^6$ -POB-dGuo were 24–250 times lower than those of  $O^2$ -POB-dThd.

In both liver and lung, total adduct levels, as well as those of  $O^2$ -POB-dThd, 7-POB-Gua and  $O^2$ -POB-Cyt, increased during the initial period of NNK treatment (Figures 3A, B; 4A, B). The maxima were observed at 10 weeks, except for  $O^2$ -POB-Cyt in lung DNA which continuously increased over time (Figure 3B). After 10 weeks, total levels of POB-DNA adducts declined gradually. The maximum amounts of POB-DNA adducts were as follows:  $O^2$ -POB-dThd, 3740 fmol/mg DNA in liver (10 weeks) and 8260 fmol/mg DNA in lung (10 weeks); 7-POB-Gua, 1460 fmol/mg DNA in liver (10 weeks) and 2220 fmol/mg DNA in lung (10 weeks);  $O^2$ -POB-Cyt: 580 fmol/mg DNA in liver (10 weeks) and 940 fmol/mg DNA (20 weeks) in lung. In lung DNA, the levels of  $O^6$ -POB-dGuo were steady during the first 10 weeks, and then declined slightly (Figure 3C). Total adduct levels in rat lung were 1.6–1.9 times higher than those in liver during the period of NNK treatment.

### POB-DNA adducts in (*R*)- and (*S*)-NNAL-treated rats

POB-DNA adducts were formed in liver and lung DNA of both (*R*)- and (*S*)-NNAL-treated rats (Figure 5A–D), with total adduct levels produced from (*S*)-NNAL significantly higher than those from (*R*)-NNAL at each time point ( $P < 0.05$ ) (Figure 6A–F). Averaging over the time points for each individual POB-DNA adduct, levels of adducts from (*S*)-NNAL treatment were also significantly greater than those from (*R*)-NNAL treatment in liver and lung ( $P < 0.05$ ), except there were too few measurable values for  $O^2$ -POB-Cyt in the (*R*)-NNAL group to do a comparison. The amounts of POB-DNA adducts in NNAL-treated rats are summarized in

Figures 4A-B and 6A-F (also see Tables 1B, C and Tables 2B, C in the Supporting Information). In liver DNA, treatment with (*S*)-NNAL for one week produced 1890 fmol/mg DNA of total POB-DNA adducts while (*R*)-NNAL only gave 110 fmol/mg DNA. Similarly, treatment with (*S*)-NNAL for one week generated 2810 fmol/mg DNA of POB-DNA adducts in lung DNA while (*R*)-NNAL gave 190 fmol/mg DNA. At various intervals, (*S*)-NNAL gave 11–17 times more POB-DNA adducts than (*R*)-NNAL in liver DNA and 6–14 times more in lung DNA (Figure 4A, B). Consistent with the results of NNK treatment, the amounts of *O*<sup>2</sup>-POB-dThd were the highest among the four POB-DNA adducts in liver and lung DNA from both the (*R*)-NNAL and (*S*)-NNAL groups. In rats treated with (*S*)-NNAL, *O*<sup>2</sup>-POB-dThd accounted for 52.4–74.2% of total adduct levels in liver and 50.2–76.1% in lung. In rats treated with (*R*)-NNAL, *O*<sup>2</sup>-POB-dThd contributed 60.9–83.9% of total adduct levels in liver and 51.8–78.8% in lung. The percentages of *O*<sup>2</sup>-POB-dThd increased over time. Similar to the results in the rats treated with NNK, the orders of the relative adduct levels in lung and liver DNA were: *O*<sup>2</sup>-POB-dThd > 7-POB-Gua > *O*<sup>2</sup>-POB-Cyt > *O*<sup>6</sup>-POB-dGuo for both (*R*)- and (*S*)-NNAL (Figure 6A-F). In rats treated with (*S*)-NNAL, levels of individual adducts were significantly different from each other ( $P < 0.01$ ), except for levels of 7-POB-Gua and *O*<sup>2</sup>-POB-dThd in liver at week one and levels of 7-POB-Gua and *O*<sup>2</sup>-POB-Cyt in lung at week 20. In rats treated with (*R*)-NNAL, there was a significant difference between levels of *O*<sup>2</sup>-POB-dThd and 7-POB-Gua in liver at weeks 5 and 20, while significant differences were observed among all adducts in lung at each time point ( $P < 0.01$ ). Comparisons among individual POB-DNA adduct levels in the (*R*)-NNAL and (*S*)-NNAL groups are shown in Table 4B, C in the Supporting Information. *O*<sup>6</sup>-POB-dGuo was detected only in lung DNA and the levels were the lowest among the four adducts at each time point. The levels of *O*<sup>6</sup>-POB-dGuo were 33–263 times lower than those of *O*<sup>2</sup>-POB-dThd in DNA of the rats treated with (*R*)-NNAL and 28–320 times lower in those treated with (*S*)-NNAL. In the (*R*)-NNAL group, total adduct levels increased over time until the maximum was found at 10 weeks with total adduct levels of 340 fmol/mg DNA in liver and 1040 fmol/mg DNA in lung (Figure 4A–B). However, for (*S*)-NNAL, the maximum total adduct levels were reached at 16 weeks with 3680 fmol/mg DNA in liver and 8730 fmol/mg DNA in lung (Figure 4A–B). For both (*R*)-NNAL and (*S*)-NNAL, total adduct levels in lung DNA were higher than those in liver DNA. (*R*)-NNAL treatment generated 1.8–3.1 times more POB-DNA adducts in lung than liver, and (*S*)-NNAL gave 1.5–2.6 times more in lung than liver.

### Comparison of POB-DNA adduct levels in NNK, (*R*)-NNAL and (*S*)-NNAL-treated rats

In both liver and lung, there was no significant difference between the total amounts of POB-DNA adducts produced from (*S*)-NNAL and those from NNK, and total adduct levels of both NNK and (*S*)-NNAL were significantly higher than those of (*R*)-NNAL ( $P < 0.05$ ) (Figure 4A, B). Except for 7-POB-Gua and *O*<sup>2</sup>-POB-Cyt in liver and *O*<sup>6</sup>-POB-dGuo in lung, the amounts of individual POB-DNA adducts were not significantly different in rats treated with NNK and (*S*)-NNAL, while levels of each individual adduct in NNK and (*S*)-NNAL-treated rats were significantly higher than those in the (*R*)-NNAL group ( $P < 0.05$ ). After the first week of treatment, total adduct levels in the (*S*)-NNAL group were 1.4 times higher than those in the NNK group in liver, and 1.3 times higher in lung (Figure 7A). The ratios of total adduct levels from (*S*)-NNAL to those from NNK decreased slowly with continuous treatment. At 10 weeks, the lowest ratios of 0.6 and 0.7 were found in lung and liver, respectively. Then the ratios remained steady at 1.0 for lung and 0.7 for liver during the remainder of the experiment. In contrast to (*S*)-NNAL, the ratios of total adduct levels from (*R*)-NNAL to those from NNK were relatively low: 0.05–0.08 for liver and 0.07–0.09 for lung during the experiment (Figure 7B).

## Discussion

DNA pyridyloxobutylation was previously demonstrated in NNAL-treated rats by quantitation of HPB-releasing DNA adducts, suggesting that the carcinogenicity of NNAL was due in part to reconversion to NNK (23). Numerous *in vitro* and *in vivo* metabolism studies confirmed the reoxidation of NNAL to NNK (18–21,23,29–30). Compared to (*R*)-NNAL, (*S*)-NNAL appeared to preferentially persist in lung and was more extensively converted to NNK in rats (20,21). Since POB-DNA adducts are entirely generated from metabolic activation of NNK, not from NNAL, the formation of those adducts was expected to be greater from (*S*)-NNAL treatment than from (*R*)-NNAL treatment. In the current study, the formation of POB-DNA adducts was demonstrated for the first time in liver and lung DNA isolated from (*R*)-NNAL and (*S*)-NNAL-treated rats. Consistent with our hypothesis, levels of POB-DNA adducts formed in rats treated with (*S*)-NNAL were not significantly different from those from NNK treatment, and both were significantly higher than those from (*R*)-NNAL treatment ( $P < 0.05$ ) (Figure 4A, B). The ratios of total adduct levels from (*R*)-NNAL to those from NNK ((*R*)-NNAL/NNK) were relatively low during the period of treatment, 0.05–0.08 in liver and 0.07–0.09 in lung, suggesting that less than 9% of (*R*)-NNAL was converted to NNK *in vivo* (Figure 7B). The (*S*)-NNAL/NNK DNA adduct ratio in our study was 0.6–1.4 (Figure 7A). After one-week of treatment, (*S*)-NNAL gave 1.3–1.4 times more adducts than NNK. Since conversion of NNAL to NNK is not as efficient as reduction of NNK to NNAL, and the conversion efficiency could not be greater than 100%, the results indicate that (*S*)-NNAL must be sequestered in tissues, and gradually released and converted to NNK locally. During the first week, the local concentrations of NNK, converted from sequestered (*S*)-NNAL, were apparently higher than those arising from NNK treatment. The ratios decreased over time reaching a minimum of 0.6 or 0.7, and then remained steady at 1.0 in lung and 0.7 in liver to the end of the experiment, suggesting that (*S*)-NNAL was preferentially retained in lung compared to liver. These results are consistent with those of previous studies, in which (*S*)-NNAL was found to be stereoselectively retained in rat tissues, preferentially in lung (20,21). The levels of total adducts as well as individual adducts from (*R*)-NNAL treatment reached a maximum at 10 weeks. However, (*S*)-NNAL adducts reached a maximum at 16 weeks (Figure 4A, B). The delay may also be due to tissue retention of (*S*)-NNAL and gradual release of (*S*)-NNAL from binding sites. Collectively, the results of this study are consistent with a scenario in which (*S*)-NNAL accumulates in tissues, primarily in the lung, by binding to unknown receptors, then is gradually released from binding sites and converted to NNK, followed by further bioactivation of NNK to produce POB-DNA adducts. We propose that this mechanism is critical for the lung carcinogenicity of NNK and NNAL in rodents.

In a previous study, we determined the formation of individual POB-DNA adducts in liver and lung DNA isolated from rats treated with relatively high doses of NNK, 5.2 or 20.7 mg/kg/day for 4 days by *s.c.* injection (15). Total adduct levels were 4–6 times higher in liver than in lung. The results were fully consistent with previous high-dose studies which measured released HPB as an indication of POB-DNA adduct formation (6,31–32). In the current study, the rats were chronically treated with lower doses of NNK. The dose of 10 ppm in the drinking water was comparable to 470–1200  $\mu\text{g}/\text{kg}/\text{day}$ , considering the daily water consumption and body weights. Total adduct levels were 1.6–1.9 times higher in lung than in liver. Fully consistent with NNK treatment, both (*R*)-NNAL and (*S*)-NNAL administration gave more POB-DNA adducts in lung than liver. The results agree with previous studies, in which HPB-releasing DNA adducts were measured at low NNK doses. Murphy et al. found that the levels of HPB released from acid hydrolysis of lung DNA were greater than from liver DNA in rats treated by *i. p.* administration of 15–150  $\mu\text{g}$  NNK/kg/day for four days (31). Similar results were obtained by Boysen et al in rats treated with 2 ppm of NNK in the drinking water for 8 and 16 weeks (9).

In calf thymus DNA modified with 4-(acetoxymethylnitrosamino)-1-(3-pyridyl)-1-butanone (NNKOAc, **3**), a chemically activated form of NNK, levels of 7-POB-Gua were the highest among four POB-DNA adducts, indicating the highest reactivity of Gua in DNA toward pyridyloxobutylating agent **9** in vitro (15). In the current study, *O*<sup>2</sup>-POB-dThd was the most abundant adduct in liver and lung of rats treated with NNK at each time point (Figure 3A, B). The differences were significant ( $P < 0.01$ ) except that the level of *O*<sup>2</sup>-POB-dThd was not significantly different from that of 7-POB-Gua in liver at the first week of treatment. The percentages of *O*<sup>2</sup>-POB-dThd of total adducts (49.6–70.0% in liver and 51.1–73.4% in lung DNA) increased over time, while the percentages of 7-POB-Gua decreased (18.7–39.1% in liver DNA and 15.0–35.5% in lung DNA). The difference may be due to the different efficiency of in vivo cellular repair systems for *O*<sup>2</sup>-POB-dThd and 7-POB-dGuo. It appears that *O*<sup>2</sup>-POB-dThd was poorly repaired in vivo and accumulated over time, while 7-POB-dGuo was relatively efficiently removed. Furthermore, 7-POB-dGuo could also be partially lost due to spontaneous loss of HPB or depurination under physiological conditions or during DNA isolation. Levels of 7-POB-Gua were previously shown to be similar to those of *O*<sup>2</sup>-POB-dThd in the high dose study, which may result from the different dose regimen used (15). The short-term treatment and higher dose could result in the saturation of repair of 7-POB-dGuo resulting in its accumulation. These results support our previous conclusion that *O*<sup>2</sup>-POB-dThd could be a useful biomarker for tobacco carcinogenesis studies (15).

Although the miscoding properties of *O*<sup>2</sup>-POB-dThd are unknown, its analog, *O*<sup>2</sup>-ethyl-dThd, was shown to be mutagenic, causing T to A transversions (33,34). The highly abundant *O*<sup>2</sup>-POB-dThd may be important in NNK carcinogenicity and mutagenicity. An in vitro miscoding study of *O*<sup>2</sup>-POB-dThd would be helpful to address this question.

*O*<sup>6</sup>-POB-dGuo was detected only in lung. Its levels were 24–250 times lower than those of *O*<sup>2</sup>-POB-dThd, indicating efficient repair of *O*<sup>6</sup>-POB-dGuo in vivo. Levels of *O*<sup>6</sup>-POB-dGuo in lung were steady for the first 10 weeks, then declined slightly, indicating that the concentrations of *O*<sup>6</sup>-POB-dGuo reached a steady state between formation and repair in lung. The results agree with our previous study, in which *O*<sup>6</sup>-POB-dGuo was shown to be efficiently repaired in rat liver and lung. These results demonstrate the persistence of the mutagenic *O*<sup>6</sup>-POB-dGuo in rat lung and support the hypothesis that *O*<sup>6</sup>-POB-dGuo is important for NNK lung carcinogenesis in rats (15). In the rats treated with (*R*)- and (*S*)-NNAL, the relative adduct levels shared the same trends as in those treated with NNK: *O*<sup>2</sup>-POB-dThd > 7-POB-Gua > *O*<sup>2</sup>-POB-Cyt > *O*<sup>6</sup>-POB-dGuo, consistent with POB-DNA adduct formation *via* conversion of (*R*)- or (*S*)-NNAL to NNK.

In both liver and lung, the maximal levels of total POB-DNA adducts were found at 10 weeks for NNK and (*R*)-NNAL and at 16 weeks for (*S*)-NNAL, then decreased slowly (Figure 4A, B). During the 20-week experiment, the body weight of rats increased, while water consumptions decreased slightly. Less carcinogen intake per gram body weight of rats could slightly contribute to the decreased adduct levels at later time points, but it is not the main reason because similar results were found in a previous study, in which rats were given s.c. injections of NNK (in 0.9% saline) at a dose of 1.76 mg/kg three times a week for 4, 8, 12, 16 or 20 weeks (6). POB-DNA adducts in lung, as quantified by HPB released from DNA, doubled from 4 to 12 weeks, then declined from 12 to 20 weeks. The results of this study were also similar to those from a previous study of HPB-releasing hemoglobin adducts resulting from 2 ppm NNK administration to rats in drinking water in a 111-week course of treatment (35, 36). The maximum was reached at 16 weeks, and then adduct levels decreased during the remaining course of the experiment. A similar trend was also observed in another previous study of HPB-releasing hemoglobin adducts, in which rats were treated with 2 ppm of NNK in the drinking water for 16 weeks. The maximal amount of HPB-releasing hemoglobin adducts was found at 12 weeks and then the levels decreased between 12 and 16 weeks (9). These



adduct level patterns could result from the effects of chronic NNK treatment on enzymes involved in NNK metabolic activation and detoxification (29).

$\alpha$ -Methylene hydroxylation of NNK and NNAL produces the same methyl-DNA adducts (Scheme 1) (1). A number of studies demonstrated that DNA methylation is important for NNK tumorigenesis in A/J mouse lung (37,38). A study with deuterium-substituted NNK and NNAL analogues demonstrated that DNA methylation is necessary for lung tumorigenicity (37). In another study, a strong correlation between lung tumor yields and levels of  $O^6$ -methylGua at 96 h after various doses of NNK indicated that  $O^6$ -methylGua is a critical determinant for NNK lung tumorigenicity in A/J mice (38). Mutagenic  $O^6$ -methylGua may also contribute to NNK tumorigenicity in rats since it accumulated and persisted in NNK-treated rats (7,39). Besides reconversion of NNAL to NNK, direct  $\alpha$ -methyl hydroxylation of NNAL, which produces PHB-DNA adducts, may also be an important mechanism for NNK and NNAL carcinogenesis in rodents. Determination of levels of methyl-DNA adducts and PHB-DNA adducts in rat liver and lung DNA used in this study would provide useful information about the relative contributions of various bioactivation pathways to NNK and NNAL carcinogenesis in rats. These studies are currently underway in our laboratory.

In summary, chronic treatment of rats with NNK, (*R*)-NNAL or (*S*)-NNAL at low doses gave higher levels of POB-DNA adducts in lung than in liver.  $O^2$ -POB-dThd was the major POB-DNA adduct found in vivo and accumulated over the course of treatment. The highly abundant  $O^2$ -POB-dThd may be important for NNK and NNAL carcinogenicity.  $O^6$ -POB-dGuo was found to persist in lung, supporting its important role in NNK and NNAL lung carcinogenesis in rats. This is the first study to examine the formation of NNK-derived POB-DNA adducts in (*R*)- and (*S*)-NNAL-treated rats. (*S*)-NNAL generated comparable levels of adducts as NNK, significantly higher than (*R*)-NNAL. The results support the hypothesis that preferential retention of (*S*)-NNAL in the lung, followed by reconversion to NNK, then metabolic activation of NNK, is critical for lung carcinogenesis by NNK and NNAL in rodents.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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## Abbreviations

### ANOVA

analysis of variance

### HPB

4-hydroxy-1-(3-pyridyl)-1-butanone

### HPLC-ESI-MS/MS

high performance liquid chromatography-electrospray ionization-tandem mass spectrometry

### 11 $\beta$ -HSD1

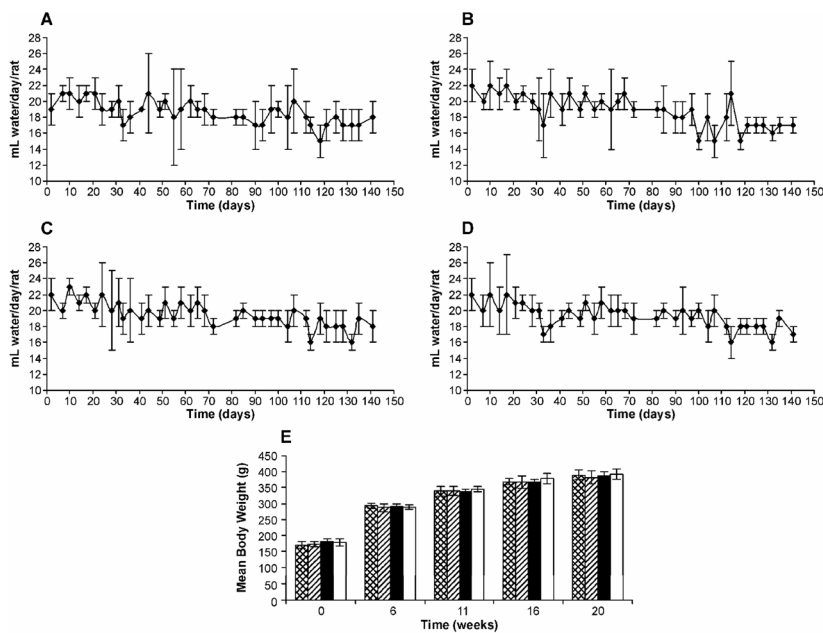
	11 $\beta$ -hydroxysteroid dehydrogenase type 1
<b>NNK</b>	4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone
<b>NNAL</b>	4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol
<b>NNN</b>	<i>N'</i> -nitrosonornicotine; <i>O</i> <sup>2</sup> -POB-dCyd, <i>O</i> <sup>2</sup> -[4-(3-pyridyl)-4-oxobut-1-yl]-2'-deoxycytidine; <i>O</i> <sup>2</sup> -POB-Cyt, <i>O</i> <sup>2</sup> -[4-(3-pyridyl)-4-oxobut-1-yl]cytosine
<b><i>O</i><sup>2</sup>-POB-dThd</b>	<i>O</i> <sup>2</sup> -[4-(3-pyridyl)-4-oxobut-1-yl]thymidine
<b><i>O</i><sup>6</sup>-POB-dGuo</b>	<i>O</i> <sup>6</sup> -[4-(3-pyridyl)-4-oxobut-1-yl]-2'-deoxyguanosine; 7-POB-dGuo, 7-[4-(3-pyridyl)-4-oxobut-1-yl]-2'-deoxyguanosine; 7-POB-Gua, 7-[4-(3-pyridyl)-4-oxobut-1-yl]guanine
<b>PHB</b>	4-(3-pyridyl)-4-hydroxybut-1-yl
<b>POB</b>	4-(3-pyridyl)-4-oxobut-1-yl
<b>REML</b>	restricted maximum likelihood
<b>SRM</b>	selected reaction monitoring

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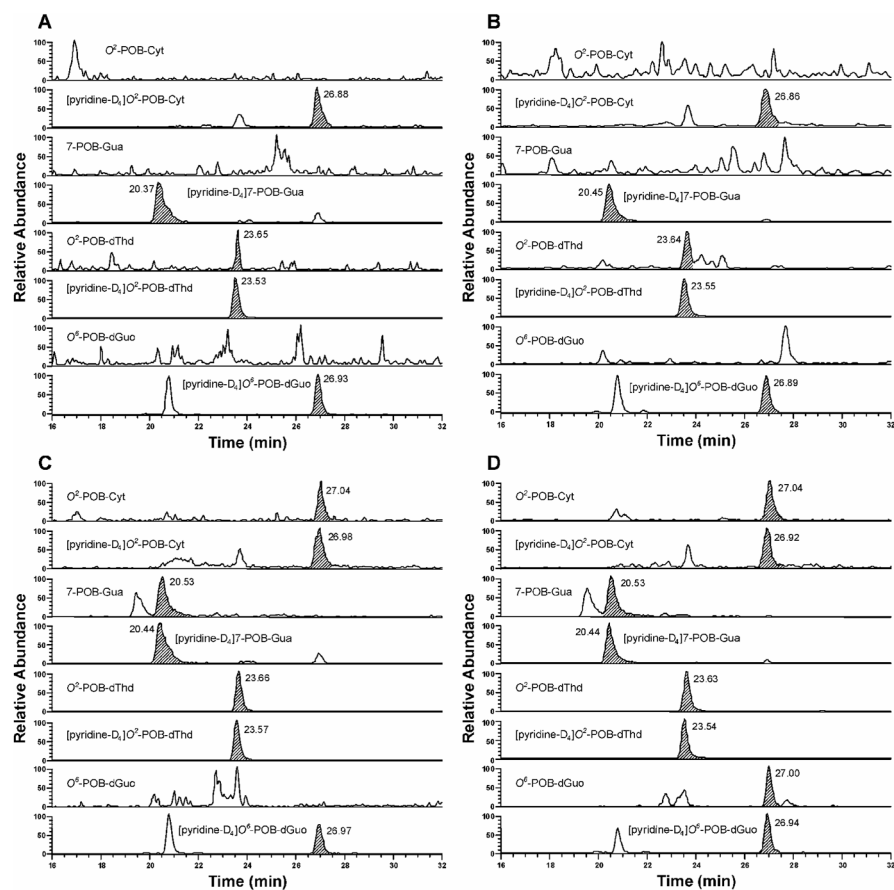
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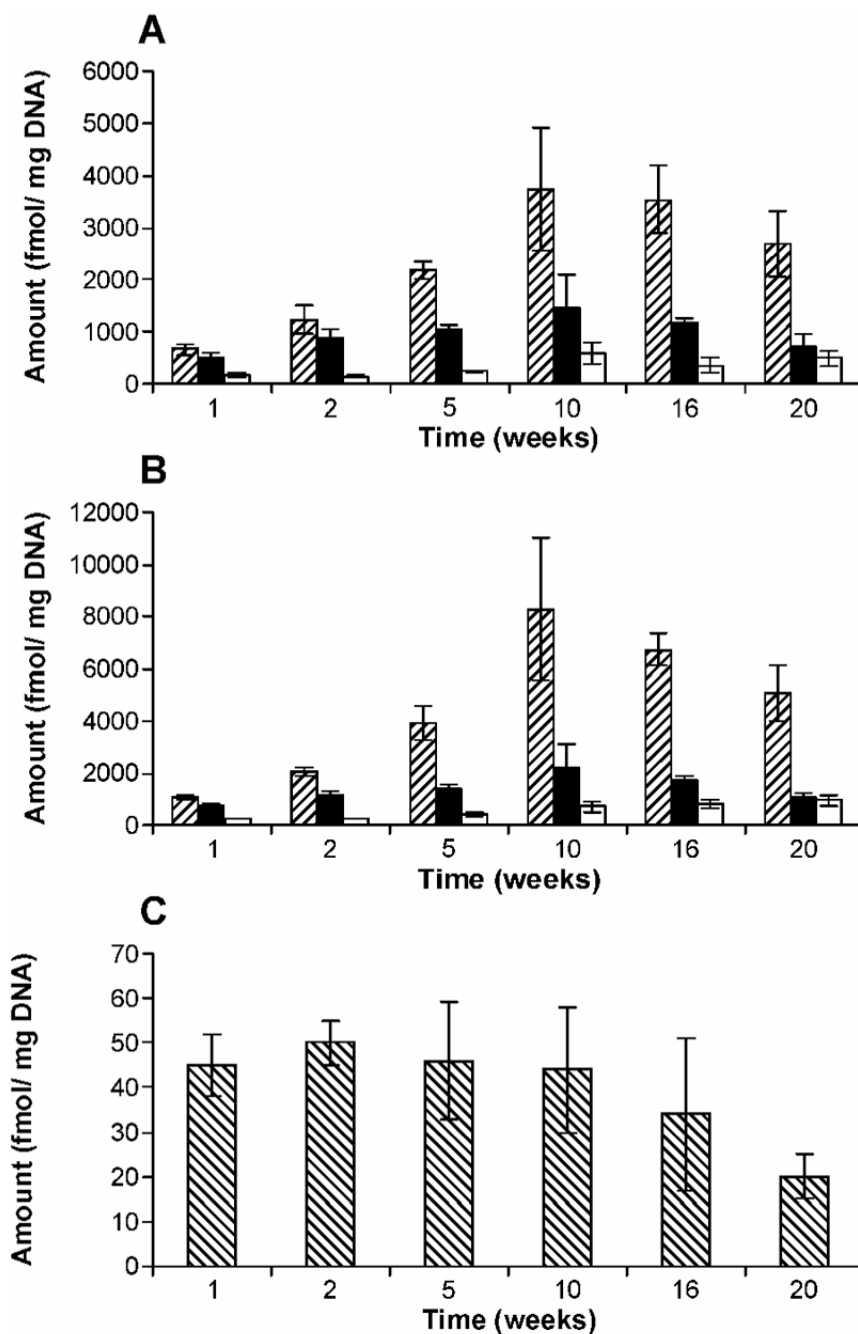
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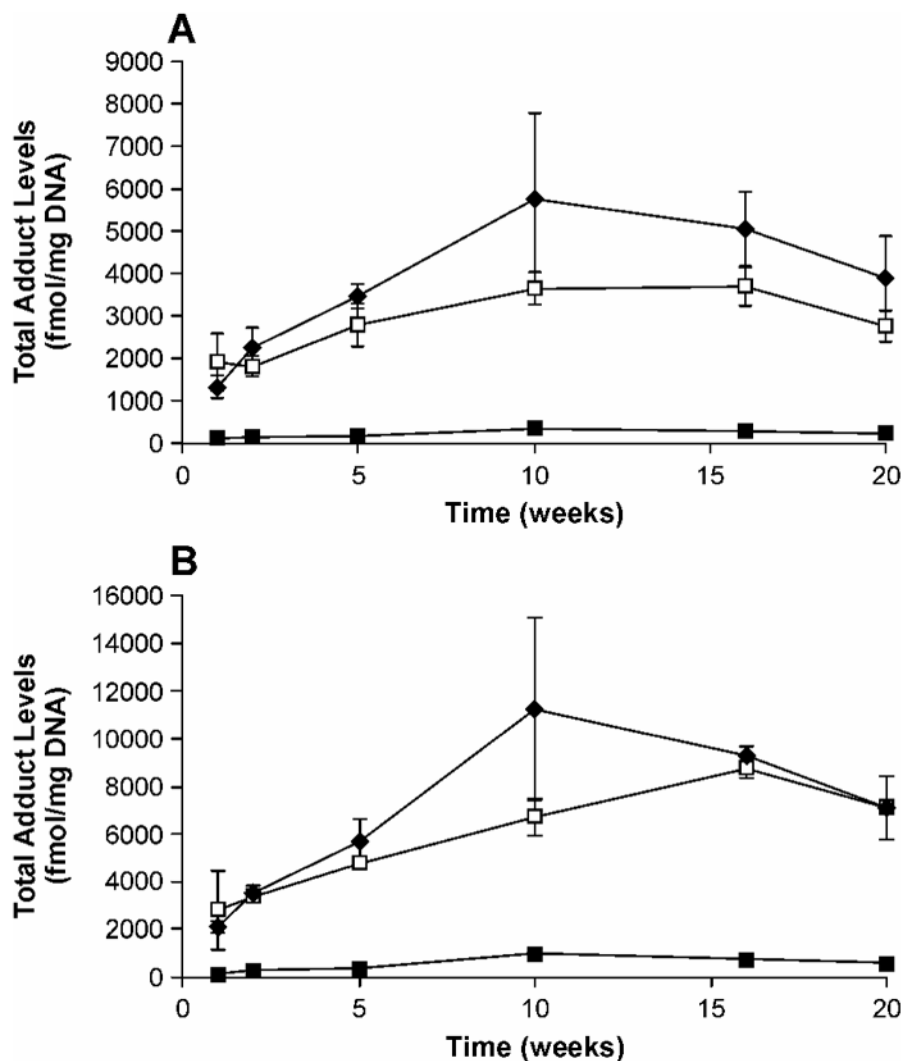
**Figure 1.** Water consumption by (A) control rats; (B) NNK-treated rats; (C) (R)-NNAL-treated rats; (D) (S)-NNAL-treated rats; and (E) body weights of rats in control, NNK, (R)-NNAL and (S)-NNAL groups during the 20-week experiment. Values for water consumption and body weights are the mean  $\pm$  S.D. of measurements of all rats in each group. Symbol designations are: ■, control; ▨, NNK; ■, (R)-NNAL; □, (S)-NNAL.



**Figure 2.** Typical SRM chromatograms obtained upon analysis of (A) liver DNA from control rats; (B) lung DNA from control rats; (C) liver DNA from NNK-treated rats; and (D) lung DNA from NNK-treated rats after 16 weeks of treatment. Individual POB-DNA adducts or internal standards were monitored as indicated on each channel. In Figure 2A and 2B, the peaks eluting at the position of  $O^2$ -POB-dThd, the areas of which are 0.2 % of [pyridine- $D_4$ ] $O^2$ -POB-dThd, originate from the internal standard.

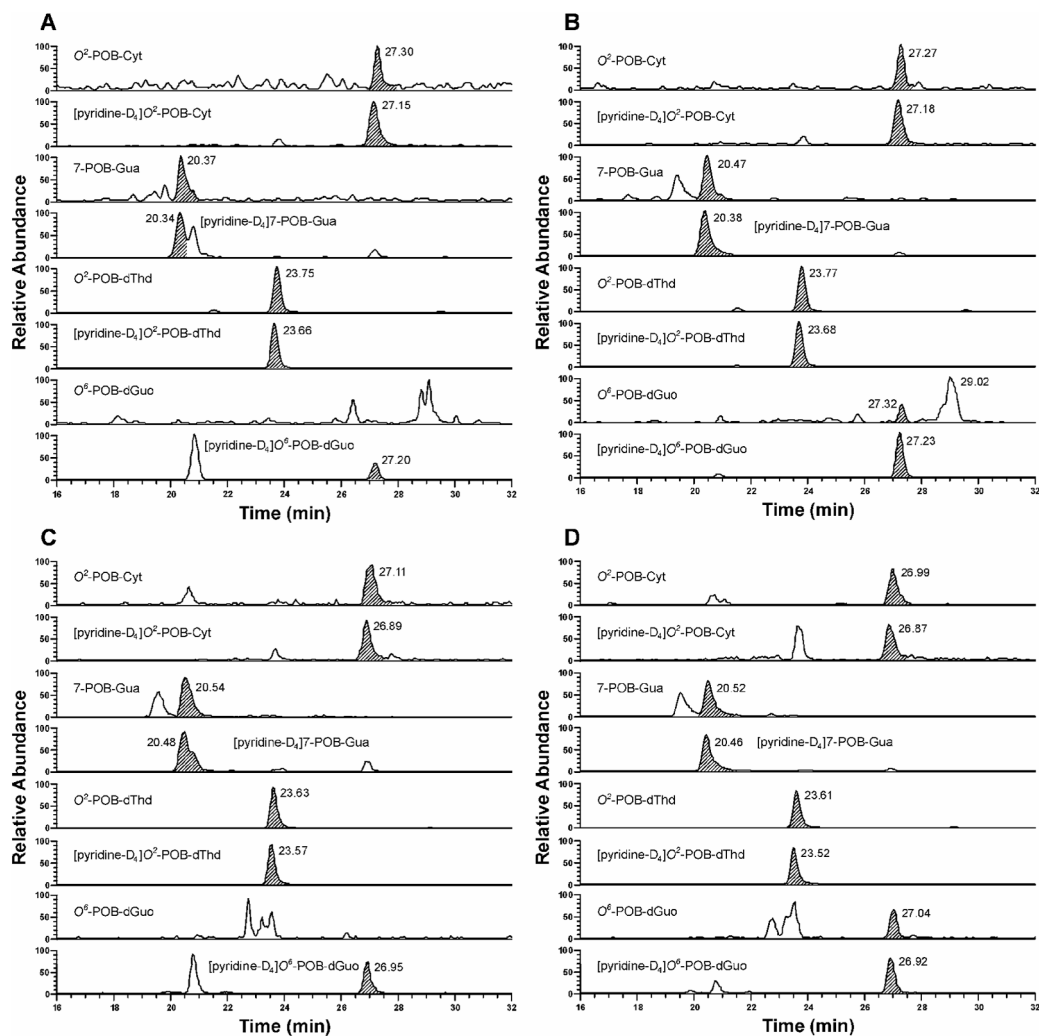


**Figure 3.** Levels of POB-DNA adducts in (A) liver and (B) lung DNA of NNK-treated rats; (C):  $O^6$ -POB-dGuo in lung DNA of NNK-treated rats on a different scale. Symbol designations are: ▨,  $O^2$ -POB-dThd; ■, 7-POB-Gua; □,  $O^2$ -POB-Cyt; ▩,  $O^6$ -POB-dGuo. Each value is the mean  $\pm$  S.D. of single analyses of DNA samples from three rats per group.

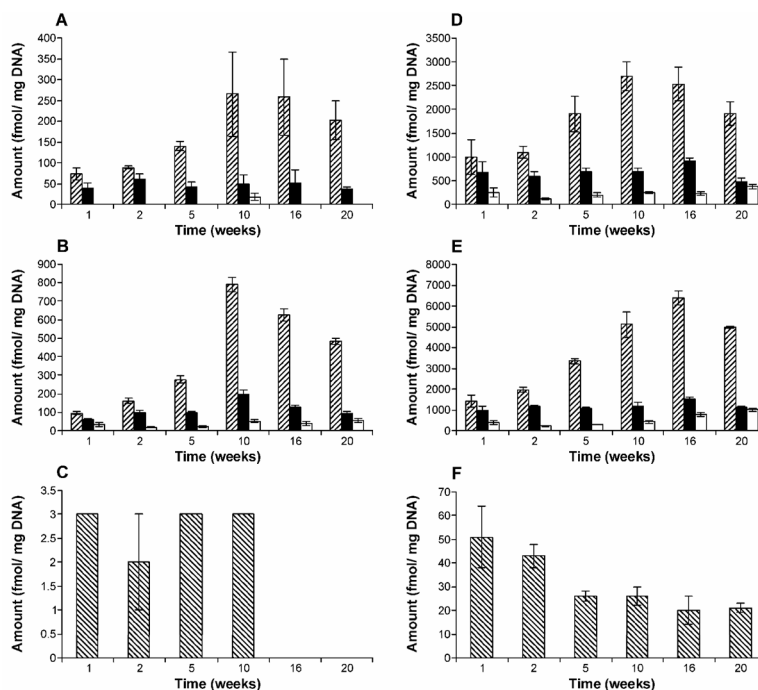


**Figure 4.** Plots of total adduct levels (fmol/mg DNA) versus time (weeks) in (A) liver and (B) lung DNA of NNK, (R)- and (S)-NNAL-treated rats. Symbol designations are:  $\blacklozenge$ , NNK,  $\square$ , (S)-NNAL;  $\blacksquare$ , (R)-NNAL. Values are the sum of amounts of all POB-DNA adducts measured in lung or liver DNA at each time point  $\pm$  S. D.

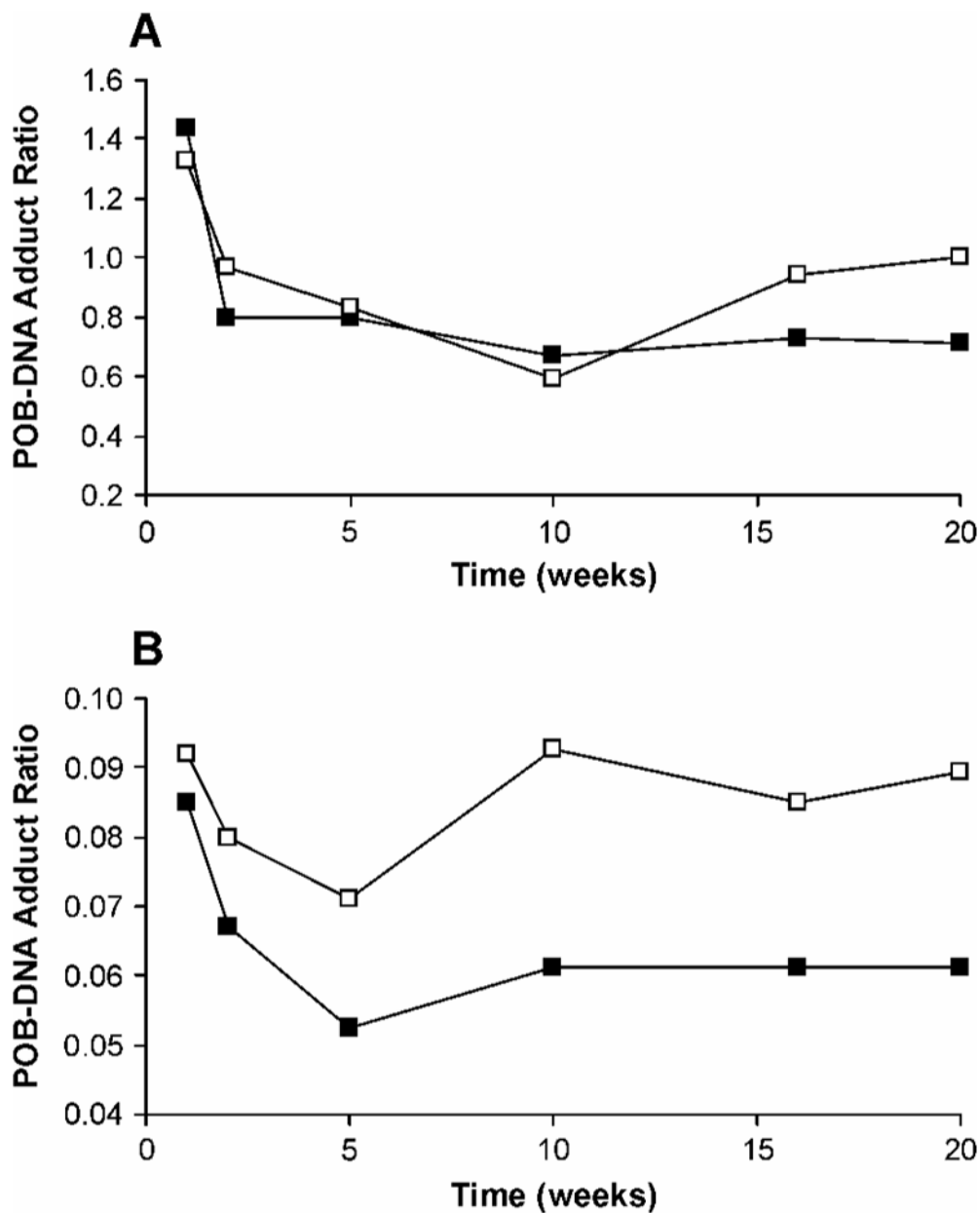




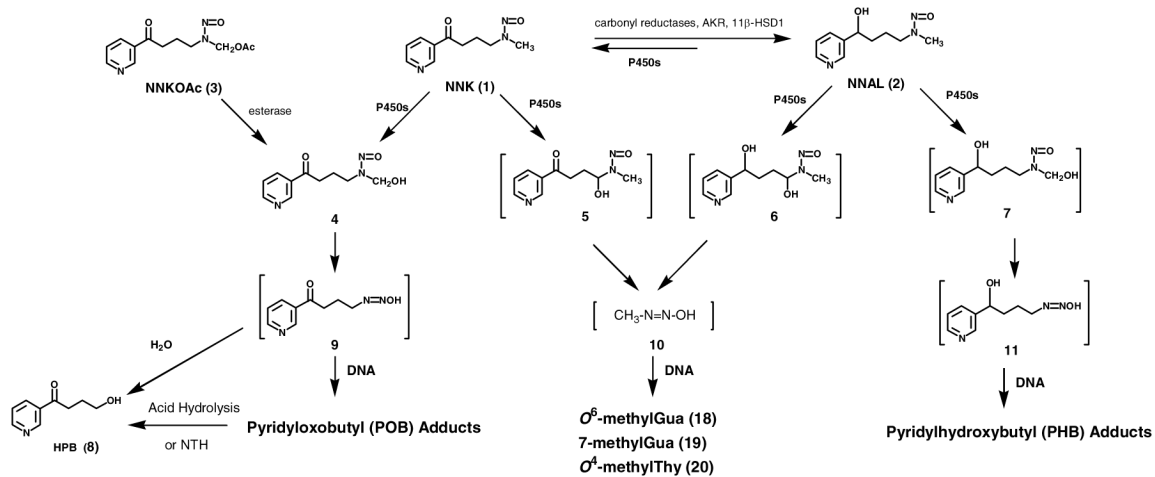
**Figure 5.** Typical SRM chromatograms obtained upon analysis of (A) liver DNA from (*R*)-NNAL-treated rats after 10 weeks of treatment; (B) lung DNA from (*R*)-NNAL-treated rats after 10 weeks of treatment; (C) liver DNA from (*S*)-NNAL-treated rats after 16 weeks of treatment; and (D) lung DNA from (*S*)-NNAL-treated rats after 16 weeks of treatment. Individual POB-DNA adducts or internal standards were monitored as indicated on each channel.



**Figure 6.** Levels of POB-DNA adducts in (A) liver and (B) lung DNA of (*R*)-NNAL-treated rats; (D) liver and (E) lung DNA of (*S*)-NNAL-treated rats; panel (C): *O*<sup>6</sup>-POB-dGuo in lung DNA of (*R*)-NNAL-treated rats on a different scale; panel (F): *O*<sup>6</sup>-POB-dGuo in lung DNA of (*S*)-NNAL-treated rats on a different scale. Symbol designations are: ▨, *O*<sup>2</sup>-POB-dThd; ■, 7-POB-Gua; □, *O*<sup>2</sup>-POB-Cyt; ▩, *O*<sup>6</sup>-POB-dGuo. Each value is the mean ± S.D. of single analyses of DNA samples from three rats per group.

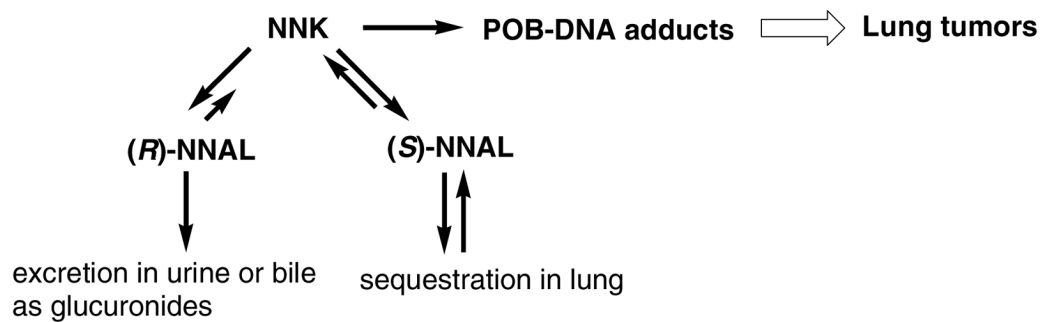


**Figure 7.** Plots of total POB-DNA adduct ratios of (A) (*S*)-NNAL to NNK and (B) (*R*)-NNAL to NNK versus time (weeks). The ratio is calculated by dividing total adduct levels from (*S*)-NNAL or (*R*)-NNAL by those from NNK. Symbol designations are: □, lung; ■, liver.

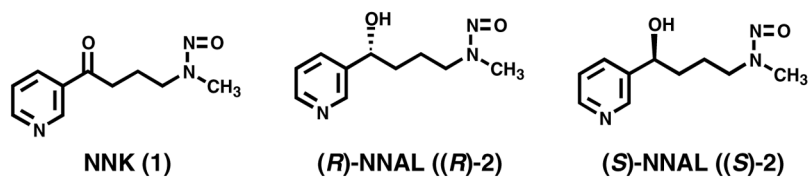


**Scheme 1.**

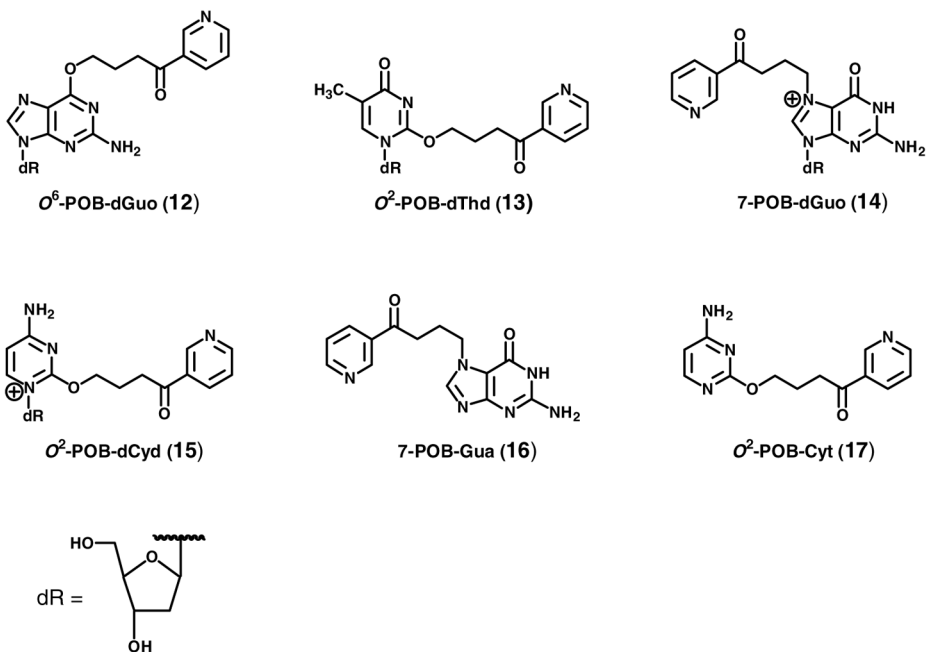
Overview of NNK and NNAL metabolism and DNA adduct formation. NTH: neutral thermal hydrolysis.



**Scheme 2.**  
Proposed mechanism for centrality of (S)-NNAL in NNK lung tumorigenesis in rodents.



**Chart 1.**  
Structures of NNK, (R)-NNAL and (S)-NNAL



**Chart 2.**  
Structures of POB-DNA adducts **12–17**. dR: 2'-deoxyribosyl.

**Table 1**

Ion transitions for SRM of POB-DNA adducts and corresponding internal standards

Adducts	Parent ion ( $[M + 1]^+$ , $m/z$ )	Ion transitions
		Daughter ion ( $m/z$ )
<i>O</i> <sup>6</sup> -POB-dGuo ( <b>12</b> )	415.1	148.1 [POB] <sup>+</sup>
[pyridine-D <sub>4</sub> ] <i>O</i> <sup>6</sup> -POB-dGuo	419.1	152.1 ([pyridine-D <sub>4</sub> ]POB) <sup>+</sup> and [Gua+H] <sup>+</sup>
<i>O</i> <sup>2</sup> -POB-dThd ( <b>13</b> )	390.1	148.1 [POB] <sup>+</sup>
[pyridine-D <sub>4</sub> ] <i>O</i> <sup>2</sup> -POB-dThd	394.1	152.1 ([pyridine-D <sub>4</sub> ]POB) <sup>+</sup>
7-POB-Gua ( <b>16</b> )	299.1	148.1 [POB] <sup>+</sup>
[pyridine-D <sub>4</sub> ]7-POB-Gua	303.1	152.1 ([pyridine-D <sub>4</sub> ]POB) <sup>+</sup> and [Gua+H] <sup>+</sup>
<i>O</i> <sup>2</sup> -POB-Cyt ( <b>17</b> )	259.1	148.1 [POB] <sup>+</sup>
[pyridine-D <sub>4</sub> ] <i>O</i> <sup>2</sup> -POB-Cyt	263.1	152.1 ([pyridine-D <sub>4</sub> ]POB) <sup>+</sup>