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Quantitation of Pyridyloxobutyl DNA Adducts in Nasal and Oral Mucosa of Rats Treated Chronically with Enantiomers of *N'*-nitrosonornicotine

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Abstract

N'-nitrosonornicotine (NNN) is one of the most important strong carcinogens in tobacco products, and is believed to play a significant role in the induction of esophageal cancer in smokers and oral cavity cancer in snuff dippers. NNN is metabolically activated through cytochrome P450-catalyzed α -hydroxylation. 2'-Hydroxylation produces a reactive intermediate 4-(3-pyridyl)-4-oxobutanediazohydroxide (**7**), which alkylates DNA to form pyridyloxobutyl (POB)-DNA adducts. DNA pyridyloxobutylation from NNN treatment, as measured by released 4-hydroxy-1-(3-pyridyl)-1-butanone (HPB, **8**), has been observed *in vitro* and *in vivo*. In the present study, we have used liquid chromatography-electrospray ionization-tandem mass spectrometry (LC-ESI-MS/MS) to analyze specific POB-DNA adducts in the nasal olfactory, nasal respiratory, and oral mucosa of F344 rats treated chronically with (*R*)-NNN or (*S*)-NNN in the drinking water (10 ppm, 1–20 weeks). Adduct levels in the nasal respiratory mucosa exceeded those in the nasal olfactory and oral mucosa. (*R*)-NNN treatment generated 2–4 times more adducts in the nasal olfactory and respiratory mucosa than did (*S*)-NNN at most time points. *O*²-[4-(3-pyridyl)-4-oxobut-1-yl]thymidine (*O*²-POB-dThd, **11**) predominated in the nasal olfactory and respiratory mucosa, followed by 7-[4-(3-pyridyl)-4-oxobut-1-yl]guanine (7-POB-Gua, **14**). Levels of *O*²-[4-(3-pyridyl)-4-oxobut-1-yl]cytosine (*O*²-POB-Cyt, **13**) and *O*⁶-[4-(3-pyridyl)-4-oxobut-1-yl]-2'-deoxyguanosine (*O*⁶-POB-dGuo, **12**) were significantly lower. In the oral mucosa, the opposite stereoselectivity was observed, with (*S*)-NNN treatment producing 3–5 times more POB-DNA adducts than did (*R*)-NNN. *O*²-POB-dThd and 7-POB-dGuo were the two major adducts, and their levels were similar. Overall, POB-DNA adduct formation in the nasal olfactory and nasal respiratory mucosa was similar to that previously observed in the lung, whereas in the oral mucosa, the trend resembled that in the esophagus. These results indicate that different mechanisms are involved in NNN metabolism and tumorigenesis in rat nasal and oral tissues. NNN enters the nasal mucosa through the circulation, and tissue-specific metabolism is important; while in the oral mucosa, direct exposure and local activation both play significant roles. Our results also support the potential importance of NNN as an oral carcinogen in people who use smokeless tobacco products.

Keywords

Pyridyloxobutyl DNA adducts; *N'*-nitrosonornicotine

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Introduction

The tobacco-specific nitrosamine *N'*-nitrosonornicotine (NNN, **1**, Chart 1) is present in substantial amounts in cigarette smoke and in unburned tobacco (1,2). Nasal mucosa and esophagus are the major target tissues of NNN carcinogenicity in rats. Tumors in both sites are observed when NNN is given in the drinking water, whereas administration of NNN by injection or gavage produces mainly nasal tumors (3). NNN also induces respiratory tract tumors in mice and hamsters, and tumors of the nasal mucosa in mink (3). A mixture of NNN and the related nitrosamine 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK, **2**), given by oral swabbing to rats, causes oral tumors (4), while NNK alone does not cause oral tumors by this route (5). These data, together with its exposure levels in tobacco products, strongly suggest that NNN is causatively related to esophageal cancer in smokers and oral cavity cancer in people who use smokeless tobacco products (1–3). NNN and NNK are considered carcinogenic to humans by the International Agency for Research on Cancer (1).

Like other tobacco-specific nitrosamines, NNN requires metabolic activation to exert its carcinogenicity. Cytochrome P450 catalyzed α -hydroxylation occurs at both the 2' and 5' positions of NNN, leading to reactive intermediates which alkylate DNA and form adducts (3). As outlined in Scheme 1, 2'-hydroxylation of NNN produces 4-(3-pyridyl)-4-oxobutanediazohydroxide (**7**), the same intermediate produced from α -methyl hydroxylation of NNK, which results in the formation of pyridyloxobutyl (POB)-DNA adducts including: *O*²-[4-(3-pyridyl)-4-oxobut-1-yl]-2'-deoxycytidine (*O*²-POB-dCyd, **9**), 7-[4-(3-pyridyl)-4-oxobut-1-yl]-2'-deoxyguanosine (7-POB-dGuo, **10**), *O*²-[4-(3-pyridyl)-4-oxobut-1-yl] thymidine (*O*²-POB-dThd, **11**), and *O*⁶-[4-(3-pyridyl)-4-oxobut-1-yl]-2'-deoxyguanosine (*O*⁶-POB-dGuo, **12**) (Chart 2) (6–8). 5'-Hydroxylation of NNN generates another intermediate **6**, which also alkylates DNA to form adducts **15** – **17**, as well as **18** and **19** (Chart 2) after NaBH₃CN reduction (9,10). Various data indicate that 2'-hydroxylation is the major metabolic activation pathway of NNN in the rat. 2'-Hydroxylation predominates in rat esophagus and nasal mucosa, major target tissues of NNN, as well as in oral tissue (11–14). POB-DNA adducts, measured by released 4-hydroxy-1-(3-pyridyl)-1-butanone (HPB, **8**) upon acid hydrolysis, have been detected in liver and nasal mucosa of NNN-treated rats, and in rat esophagus incubated with NNN (14–16).

NNN has a chiral center at its 2' position. (*S*)-NNN is the major enantiomer in tobacco products and is metabolized differently from (*R*)-NNN (17). 2'-Hydroxylation products are found predominantly from the incubation of (*S*)-NNN with cultured rat esophagus, and in the urine of (*S*)-NNN-treated rats, whereas 5'-hydroxylation is more favored in (*R*)-NNN metabolism under these conditions (18). In a previous study from our lab, a highly sensitive liquid chromatography-electrospray ionization-tandem mass spectrometry (LC-ESI-MS/MS) method was developed to analyze specific POB-DNA adducts **9**–**12**. The two unstable adducts **9** and **10** were analyzed as their bases: *O*²-[4-(3-pyridyl)-4-oxobut-1-yl]cytosine (*O*²-POB-Cyt, **13**) and 7-[4-(3-pyridyl)-4-oxobut-1-yl]guanine (7-POB-Gua, **14**) (Chart 2) after loss of deoxyribose upon neutral thermal hydrolysis (19). POB-DNA adduct formation has been investigated in liver, lung, and esophagus of rats treated chronically with 10 ppm of (*R*)-NNN or (*S*)-NNN in the drinking water (20). (*S*)-NNN treatment generated more adducts in rat esophagus and liver, whereas in the lung, more adducts formed upon (*R*)-NNN treatment. In the present study, we investigated POB-DNA adduct formation in nasal olfactory, nasal respiratory, and oral mucosa of these rats. DNA was isolated at various time points, and individual POB-DNA adducts were quantified by LC-ESI-MS/MS.

Materials and Methods

CAUTION

NNN is carcinogenic. It should be handled in a well-ventilated hood with extreme care and appropriate protective equipment.

Chemicals—(*R*)- and (*S*)-NNN, POB-DNA adducts **11–14** and internal standards were synthesized previously (17,19,21). Puregene DNA purification solutions were obtained from Qiagen (Valencia, CA). Calf thymus DNA, micrococcal nuclease (from *Staphylococcus aureus*), and phosphodiesterase II (from bovine spleen) were purchased from Worthington Biochemical Co. (Lakewood, NJ). Alkaline phosphatase (from calf intestine) was procured from Roche Diagnostics Corporation (Indianapolis, IN). All other chemicals were obtained from Sigma-Aldrich.

Animal Experiment—This was the same experiment as described in our previous study (20). Briefly, 162 male F344 rats were randomly divided into three groups of 54 rats: (1) control; (2) (*R*)-NNN; and (3) (*S*)-NNN. 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. Nine rats per group were sacrificed by CO₂ overdose at 1, 2, 5, 10, 16, and 20 weeks. Tissues were harvested and stored at –80 °C until DNA isolation.

Isolation of nasal olfactory and respiratory mucosa—This was performed as previously described (15). After the rats were sacrificed, the mandibula of the head was removed. The head was placed on the necropsy board with the palate facing up. The head was split using a bone mallet and a scalpel, cutting longitudinally on the median line, through the hard palate. Respiratory mucosa was retrieved from the naso- and maxilloturbinates, the lateral walls of the nasal passages, and the median septum anterior to the olfactory area. Olfactory mucosa was obtained from the ethmoturbinates and the lateral wall and septum of the olfactory area. The tissues were stored in PBS buffer at –80 °C.

Quantitation of POB-DNA adducts by LC-ESI-MS/MS—This was performed as previously described (19), with some modifications. DNA was isolated following the Puregene DNA isolation protocol (Qiagen) (22). Nasal olfactory, nasal respiratory, and oral mucosa from nine rats in each group were divided into three pools of three rats for DNA isolation. Depending on the starting amount of tissue, the reagents were scaled accordingly. The DNA (0.1 – 1 mg), plus four deuterated internal standards of POB-DNA adducts, was subjected to neutral thermal hydrolysis (100 °C, 30 min), followed by enzymatic hydrolysis with micrococcal nuclease, phosphodiesterase II, and alkaline phosphatase. A 10 µL aliquot was removed for dGuo quantitation, and the remaining hydrolysate was purified on a solid phase extraction (SPE) cartridge [Strata-X, 33 µm, 30 mg/1 mL (Phenomenex, Torrance, CA)]. After the sample was applied, the cartridge was washed with 2 mL H₂O and 1 mL 10% CH₃OH/H₂O, and the analytes were eluted with 2 mL CH₃OH. The eluants were evaporated to dryness, and dissolved in 20 µL of 2% NH₄OAc. An 8 µL aliquot was analyzed by LC-ESI-MS/MS for POB-DNA adducts. A buffer control which lacked DNA and a calf thymus DNA sample were prepared each time and processed in the same way as negative controls.

LC-ESI-MS/MS analysis was carried out with an Agilent 1100 capillary flow HPLC (Agilent Technologies, Palo Alto, CA) equipped with a Luna 250 mm × 0.5 mm 5 µm C18 column (Phenomenex) and coupled to a Discovery Max (ThermoElectron, San Jose, CA) triple quadrupole mass spectrometer. The solvent elution program was a gradient from 5 to 65% CH₃OH in 15 mM NH₄OAc buffer in 30 min at a flow rate of 10 µL/min at 30 °C. The ESI source was operated in the positive ion mode. The adducts were analyzed by MS/MS using

selected reaction monitoring (SRM). Ion transitions and their collision energies are listed in Table 1. Other MS parameters were optimized to achieve maximum signal intensity. Calibration curves were constructed before each analysis using standard solutions containing varying amounts of each adduct with a constant amount of the corresponding deuterated internal standard in 2% NH₄OAc. The amount of DNA was calculated from the dGuo content as determined by HPLC (19), considering that 1 mg of DNA contains 3 μ mol of nucleotides (23), whereas dGuo accounts for 22% of the total nucleotides in rat DNA, as previously determined in our lab. Adduct levels were expressed as fmol adduct/mg DNA.

Statistical Analyses—Because of a highly skewed distribution, the amounts of POB-DNA adducts were transformed to the natural log scale. The following three statistical analyses were performed. (1) A repeated measures analysis of variance (ANOVA) was used to compare total POB-DNA adduct levels in three different tissue types. Tissue type was the repeated factor and time was the fixed effect. The model assumes a linear trend over time. If this global test across all six time points was significant for tissue type, then the least-squares means for the three tissues, derived from the ANOVA model, were compared two at a time for each time point separately. The Tukey procedure was utilized to control for multiple comparisons between the tissues. The above analysis was performed for (*R*)-NNN and (*S*)-NNN treatment separately. (2) An analysis of covariance (ANCOVA) was utilized to compare total POB-DNA adduct levels between the two treatments ((*R*)-NNN and (*S*)-NNN). The grouping factor was treatment and the covariate was time. The model assumes a linear trend over time. If this overall test was significant for treatment, then the least-squares means for the treatments (based on the ANCOVA model) were compared at each time point separately. (3) A repeated measures ANOVA was employed to compare individual POB-DNA adducts for each tissue and treatment. Time points were included in the model as a fixed effect. The analysis was carried out separately for each combination of tissue and treatment. If an adduct was missing or not detected for all or most of the data for a particular tissue and treatment, it was excluded for that analysis. If the global test including adducts and time points was significant, then additional tests were performed to compare the least-squares means for pairs of individual adducts at each time point. The Tukey procedure was applied to control for multiple comparisons between the adducts evaluated two at a time. Statistical significance was set at $P < 0.05$.

Results

POB-DNA adducts were not found in the nasal olfactory, nasal respiratory, and oral mucosa DNA from control rats, but were readily detected in DNA from (*R*)- and (*S*)-NNN-treated rats. Representative chromatograms from the analyses of POB-DNA adducts are shown in Figure 1. Peaks corresponding to the retention times of *O*²-POB-Cyt, 7-POB-Gua and *O*²-POB-dThd were observed, and each coeluted with internal standards. *O*⁶-POB-dGuo was not detected in these samples.

Levels of total and individual POB-DNA adducts in the nasal olfactory and respiratory mucosa of (*R*)- and (*S*)-NNN-treated rats are shown in Figure 2A and B, and Figure 3A, B, D, and E (also see Table 1S in the Supporting Information). Two adducts –7-POB-Gua and *O*²-POB-dThd – were detected in all the samples, while *O*²-POB-Cyt was seen in most samples, and *O*⁶-POB-dGuo was seldom seen, and then in very small amounts. Total adduct levels were higher in the respiratory mucosa than in the olfactory mucosa for both (*R*)- and (*S*)-NNN-treated rats, and the differences were significant at all time points ($P < 0.05$). In the respiratory mucosa, the maximum was 6410 fmol/mg DNA for the (*R*)-NNN group, observed at 10 weeks, and 3150 fmol/mg DNA for the (*S*)-NNN group, observed at 16 weeks. In comparison, the maximum in the olfactory mucosa was 1570 fmol/mg DNA for the (*R*)-NNN group, observed at 10 weeks, while for the (*S*)-NNN group, except for the unexpected high level seen at 20 weeks (2410 fmol/mg of DNA), levels at the other time points were below 390 fmol/mg of

DNA. In general, (*R*)-NNN treatment generated 2–4 times more adducts than (*S*)-NNN treatment did, except for the olfactory mucosa at 20 weeks. The differences between the (*R*)- and (*S*)-NNN groups were statistically significant at all time points in both nasal tissues ($P < 0.05$). Individual adduct levels were in the following order: O^2 -POB-dThd $>$ 7-POB-Gua $>$ O^2 -POB-Cyt $>$ O^6 -POB-dGuo. As shown in Figure 3B, O^2 -POB-dThd accounted for 52 – 81 % of total adducts in the respiratory mucosa of (*R*)-NNN-treated rats, and its level increased during the initial period of treatment and reached a plateau after 10 weeks. 7-POB-Gua accounted for 18 – 43 % of total adducts. While its level was relatively stable throughout the time course, the percentage decreased over the time. However, levels of these two adducts were not significantly different from each other ($P > 0.05$). Levels of O^2 -POB-Cyt or O^6 -POB-dGuo were significantly lower than those of O^2 -POB-dThd or 7-POB-Gua ($P < 0.05$), accounting for less than 6 % of the total. Similar patterns were observed in the olfactory mucosa of (*R*)-NNN-treated rats (Figure 3A).

POB-DNA adduct levels in the oral mucosa of (*R*)- and (*S*)-NNN-treated rats are illustrated in Figure 2C. In contrast to the nasal olfactory and respiratory mucosa, total adduct levels were 3 – 5 times higher in the (*S*)-NNN group than in the (*R*)-NNN group, and this difference was significant at all time points ($P < 0.05$). In the (*S*)-NNN-treated rats, a gradual increase in total adducts was observed during the initial period of treatment, reaching a maximum at 10 weeks (755 fmol/mg DNA), and a slight decrease was seen afterwards. Similar trends were observed in the (*R*)-NNN-treated rats, except that the maximum level was reached at 5 weeks (222 fmol/mg of DNA). In the (*R*)- and (*S*)-NNN-treated rats, O^2 -POB-dThd and 7-POB-Gua were the two major adducts, and O^2 -POB-Cyt and O^6 -POB-dGuo were only occasionally detected (Figure 3C and F). Unlike the pattern observed in the nasal olfactory and respiratory mucosa, the levels of O^2 -POB-dThd and 7-POB-Gua in the oral mucosa were similar. Levels of 7-POB-Gua were higher than those of O^2 -POB-dThd in the (*R*)-NNN group at 1, 2, and 5 weeks, as well as in the (*S*)-NNN group at 1 and 2 weeks. However, none of these differences was statistically significant ($P > 0.05$).

Figure 4 illustrates the comparison of total POB-DNA adduct levels in various tissues of NNN-treated rats. The levels in the lung, liver, and esophagus were measured and reported previously (20). Among those tissues in which (*R*)-NNN treatment generated more adducts than (*S*)-NNN treatment, nasal respiratory mucosa had the highest POB-DNA adduct levels, followed by nasal olfactory mucosa and lung. For those tissues with opposite stereoselectivity, the following order was observed: esophagus $>$ oral mucosa $>$ liver.

Discussion

NNN is one of the most prevalent nitrosamines in tobacco products. It is present in substantial amounts in both cigarette smoke and smokeless tobacco products. 2'-Hydroxylation is considered the major pathway of NNN activation in rats. In *in vitro* studies, a clear preference for 2'-hydroxylation products was observed when NNN was incubated with rat nasal and oral tissues, and esophagus (11–14). DNA pyridyloxobutylation from 2'-hydroxylation, as measured by released HPB upon acid hydrolysis, was observed in rat esophagus incubated with [$5\text{-}^3\text{H}$]NNN (14) and in liver and nasal mucosa of rats treated with NNN (15,16). The results of this study extend these earlier results and clearly demonstrate the presence of individual POB-DNA adducts in nasal olfactory, nasal respiratory, and oral mucosa of NNN-treated rats.

Levels of these adducts depended on the tissue in which they were found. Our previous study showed that adducts from (*S*)-NNN predominated in esophagus and liver, while adducts from (*R*)-NNN were greater in lung (20). This study showed that adducts from (*S*)-NNN predominated in oral mucosa, while those from (*R*)-NNN were greater in nasal olfactory and respiratory mucosa. This tissue-related stereoselectivity of POB-DNA adduct formation by

NNN enantiomers probably results in part from the tissue distribution of NNN enantiomers after oral administration. The higher levels of adducts from (*S*)-NNN in liver indicate that (*S*)-NNN is the more extensively metabolized enantiomer in liver. Because lung and nasal mucosa are exposed to the carcinogen after hepatic clearance of the oral dose, it is likely that less (*S*)-NNN is delivered to these tissues than (*R*)-NNN. POB-DNA adducts are formed after NNN is metabolically activated at these sites. Therefore, we would expect lower levels of POB-DNA adducts in the lung and nasal mucosa from (*S*)-NNN treatment than from (*R*)-NNN treatment. Esophagus and oral mucosa, on the other hand, have direct contact with the carcinogen when NNN is administered in the drinking water, and there is no effect of hepatic clearance. Therefore, adduct formation would be dependent on direct activation of NNN after local exposure. This activation proceeds mainly by 2'-hydroxylation to POB-DNA adducts, consistent with previous findings that cultured rat esophagus metabolizes (*S*)-NNN preferentially through 2'-hydroxylation, and (*R*)-NNN preferentially through 5'-hydroxylation (18). Additional experiments are required to fully characterize the pharmacokinetics of (*R*)- and (*S*)-NNN in the rat.

NNN is a well-established esophageal carcinogen in rats (1,3). A few tongue tumors were also observed in rats treated with NNN administered orally (12). Oral cavity tumors were induced when rats were given a combination of NNK and NNN by oral swabbing (4), but swabbing of NNK alone gave only lung tumors (5). These results suggest that NNN might induce oral cavity tumors if swabbed; however, to our knowledge, this has never been reported. Our DNA adduct data support the implication from these carcinogenicity studies that NNN is possibly an oral carcinogen in rats for the following reasons. First, the same stereoselectivity of POB-DNA adduct formation from enantiomers of NNN was observed in the esophagus and oral tissue, although higher levels were detected in the esophagus. This clearly indicates similar metabolic activation pathways of NNN in these tissues. These results are consistent with a previous study by Murphy *et al.* who demonstrated similar metabolism by cultured rat oral tissues and esophagus, both of which catalyzed the α -hydroxylation of NNN more efficiently than that of NNK (14). Second, the exposure route of NNN is similar in the esophagus and oral cavity when NNN is administered in the drinking water. Both tissues have direct contact with the carcinogen. The higher incidence of esophageal tumors in rats treated with NNN through oral administration has been hypothesized to be related to direct contact (3). Therefore, like esophagus, oral cavity might be a target tissue of NNN in rats.

Levels of NNN in unburned tobacco are higher than those of any other strong carcinogen. Three brands of conventional smokeless tobacco had amounts of NNN ranging from 0.9 to 4.5 $\mu\text{g/g}$ product wet weight (24). Smokeless tobacco is considered by the International Agency for Research on Cancer to be a cause of oral and pancreatic cancer in humans (1). The uptake of NNN by snuff-dippers has been clearly demonstrated (25). Studies have also demonstrated the penetration of NNN across porcine oral mucosa, and this penetration was enhanced by both nicotine and ethanol (26,27). As discussed above, our study suggests that NNN might cause oral cavity tumors in rats. Considering the exposure levels, NNN may be important in the induction of oral cavity cancer in smokeless tobacco users. Moreover, (*S*)-NNN is the predominant enantiomer in tobacco products (17). (*S*)-NNN produced more POB-DNA adducts in the rat oral mucosa than did (*R*)-NNN, indicating (*S*)-NNN might be more carcinogenic to the rat oral cavity than (*R*)-NNN. Since reported carcinogenicity studies have all used racemic NNN, they may have underestimated the cancer risk of NNN to humans.

There is evidence for a high affinity P450 enzyme in the rat esophagus which catalyzes the 2'-hydroxylation of NNN (28). It is likely that the same P450 is also expressed in the oral tissue in lower amounts. P450 2A3 is expressed in the rat esophagus in small quantities (29); however, this P450 does not seem to play a significant role in the metabolic activation of NNN in the rat

esophagus (30,31). The identity of this P450 in rat esophagus and oral tissue needs to be further investigated.

Tumors in the nasal cavity are frequently observed in NNN-treated rats, and are independent of the route of administration (3). This suggests that unlike the esophagus, nasal mucosa is a systemic site for NNN tumorigenesis. Because NNN is delivered to the nasal cavity through the circulation, tissue-specific metabolism and activation of NNN is important to its tumorigenesis. This is supported by an *in vivo* autoradiography study in which accumulation of tissue-bound NNN and metabolites was observed in both esophagus and nasal mucosa after injection, but the levels were higher in the nasal mucosa (32). DNA pyridyloxobutylation is indicated to be important in rat nasal carcinogenesis, because NNK and NNN, both of which can pyridyloxobutylate DNA, have similar activity toward the rat nose, but *N*-nitrosodimethylamine (NDMA), which only methylates DNA, has little effect (3). Cultured rat nasal mucosa catalyzes the 2'-hydroxylation of NNN 2–3 times more efficiently than 5'-hydroxylation (11). POB-DNA adducts, when analyzed by released HPB upon acid hydrolysis, were detected in both the olfactory and respiratory mucosa of NNN-treated rats (15). The levels in the olfactory mucosa were near the detection limit, whereas higher levels were found in the respiratory mucosa. Our results are consistent with these findings. A high-affinity P450 enzyme that catalyzes the 2'-hydroxylation of NNN must be present in the nasal mucosa. P450 2A3 does not seem to be the right candidate, because it activates (*S*)-NNN exclusively through 5'-hydroxylation, and (*R*)-NNN preferentially through 2'-hydroxylation (30). In addition, P450 2A3 is expressed only in the olfactory mucosa, not in the respiratory mucosa (33). This suggests that another P450 enzyme in the nasal mucosa must be responsible for the metabolic activation of NNN, though P450 2A3 might contribute to some extent.

P450 2A3, which is abundantly expressed in the rat nasal olfactory mucosa (33), efficiently catalyzes the 5'-hydroxylation of NNN (30). Therefore, DNA adducts from 5'-hydroxylation of NNN may be present in the olfactory mucosa. A carcinogenicity study indicated that the malignant nasal tumors induced by NNN arose mainly in the olfactory portion of the nasal mucosa (34). POB-DNA adducts analyzed in this study were lower in the olfactory portion than in the respiratory portion of the nasal tissue. It will be interesting to investigate the DNA adducts derived from 5'-hydroxylation in the olfactory mucosa of NNN-treated rats. The structures of these adducts have been characterized in our lab recently as **15 – 19**, Chart 2 (8, 9). Developing a sensitive LC-ESI-MS/MS method to analyze these adducts will be useful to examine this issue.

Our group has so far analyzed POB-DNA adducts in six different tissues of NNN-treated rats. These tissues can be divided into two groups: (1) lung, nasal olfactory and nasal respiratory mucosa, in which (*R*)-NNN treatment favors POB-DNA adduct formation; (2) esophagus, liver, and oral mucosa, in which (*S*)-NNN treatment favors adduct formation. As shown in Figure 4, the order of POB-DNA adduct levels in each group is consistent with the tumorigenicity of NNN in these tissues. Lung and liver, which are non-target tissues of NNN, have the lowest amounts of POB-DNA adducts in each group, while nasal olfactory and respiratory mucosa, and esophagus, which are well-established target tissues, have higher levels of POB-DNA adducts. Carcinogenicity studies using (*R*)- and (*S*)-NNN will be useful to further assess the importance of POB-DNA adducts in the tumor induction by NNN in these tissues. The expression and regulation of P450s in various rat tissues have been reviewed (35,36); however, the specific P450s that are responsible for the metabolic activation of NNN in these rat tissues are not clear.

POB-DNA adduct levels in the olfactory mucosa of (*S*)-NNN-treated rats were higher than those of (*R*)-NNN-treated rats at 20 weeks, which was opposite to all time points before 16 weeks. There was an outlier among the three measurements from the (*S*)-NNN group at 20

weeks. Total POB-DNA adduct level of that measurement was 6430 fmol/mg DNA, and was 15–18 times higher than the other two. A large SD resulted from these measurements, as shown in Figure 2A. There are a couple of possible explanations. First, since each measurement represents a pool of three rats, some rats in that pool had a significantly higher metabolic activation rate, or were deficient in certain DNA repair mechanisms, both of which can lead to higher levels of adducts. Second, contamination from the respiratory portion might occur during the separation of nasal tissue. However, since this measurement (6430 fmol/mg DNA) was still more than twice as high as the corresponding POB-DNA adduct levels in the respiratory portion (2740 fmol/mg DNA), the second explanation is not very likely.

In summary, we have quantified POB-DNA adducts in nasal olfactory, nasal respiratory, and oral mucosa of rats treated chronically with enantiomers of NNN. (*S*)-NNN treatment generated higher adduct levels in the oral mucosa, while (*R*)-NNN treatment produced more adducts in the nasal mucosa. Our results suggest that different mechanisms are involved in NNN metabolism and tumorigenesis in nasal and oral tissues. Our study also supports the potential involvement of NNN as a causative factor for oral cavity cancer in smokeless tobacco users.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. LC-ESI-MS/MS chromatograms obtained upon the analyses of POB-DNA adducts in nasal olfactory mucosa of rats treated with (A) (*R*)-NNN; and (B) (*S*)-NNN for 16 weeks. Individual POB-DNA adducts and their internal standards were monitored as indicated on each channel. SRM transitions are summarized in Table 1.

Figure 2.

Total POB-DNA adduct levels (fmol/mg DNA) $\pm$ SD vs time (weeks) in the (A) nasal olfactory mucosa; (B) nasal respiratory mucosa; and (C) oral mucosa of (*R*)-NNN and (*S*)-NNN treated rats. Symbol designations are: ■, (*R*)-NNN and □, (*S*)-NNN.

Figure 3.

Levels of each POB-DNA adduct in the (A) nasal olfactory mucosa of (R)-NNN-treated rats; (B) nasal respiratory mucosa of (R)-NNN-treated rats; (C) oral mucosa of (R)-NNN-treated rats; (D) nasal olfactory mucosa of (S)-NNN-treated rats; (E) nasal respiratory mucosa of (S)-NNN-treated rats; and (F) oral mucosa of (S)-NNN-treated rats. Adduct levels are shown in sequence at each time point, as follows: ▨, O²-POB-Cyt; ■, 7-POB-Gua; □, O²-POB-dThd; ▩, O⁶-POB-dGuo.

Figure 4.

Comparison of total POB-DNA adduct levels (fmol/mg DNA) vs time (weeks) in (A) lung, nasal olfactory mucosa, and nasal respiratory mucosa of (*R*)-NNN treated rats; and (B) esophagus, liver, and oral mucosa of (*S*)-NNN treated rats. POB-DNA adduct levels in the lung, esophagus, and liver were measured and reported previously (20). Symbol designations are: (A) □, lung; ◆, nasal olfactory mucosa; ■, nasal respiratory mucosa; and (B) □, esophagus; ◆, liver; ■, oral mucosa.

Scheme 1.
Overview of NNN metabolism and DNA adduct formation.

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

Chart 2.
Structures of POB-DNA adducts.

Table 1

SRM transitions of POB-DNA adducts and corresponding internal standards

Adducts	Parent ion ([M + 1] ⁺ , <i>m/z</i>)	Daughter ion (<i>m/z</i>)	Collision energy (eV)
<i>O</i> ² -POB-Cyt (13)	259.1	148.1 [POB] ⁺	17
[pyridine-D ₄] <i>O</i> ² -POB-Cyt	263.1	152.1 ([pyridine-D ₄]POB) ⁺	17
7-POB-Gua (14)	299.1	148.1 [POB] ⁺	17
[pyridine-D ₄]7-POB-Gua	303.1	152.1 ([pyridine-D ₄]POB) ⁺ and [Gua + H] ⁺	17
<i>O</i> ² -POB-dThd (11)	390.1	148.1 [POB] ⁺	20
[pyridine-D ₄] <i>O</i> ² -POB-dThd	394.1	152.1 ([pyridine-D ₄]POB) ⁺	20
<i>O</i> ⁶ -POB-dGuo (12)	415.1	148.1 [POB] ⁺	20
[pyridine-D ₄] <i>O</i> ⁶ -POB-dGuo	419.1	152.1 ([pyridine-D ₄]POB) ⁺ and [Gua + H] ⁺	20