Dose-Response Study of DNA and Hemoglobin Adduct Formation by 4-(Methylnitrosamino)-1-(3-pyridyl)-1-butanone in F344 Rats

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ABSTRACT

Levels of hemoglobin adducts and DNA adducts were measured in F344 rats after 4 consecutive daily i.p. injections of 4-(methyltriazeno)-1-(3-pyridyl)-1-butanone (NNK). The dose range was from 3 to 10,000 µg/kg/day. [5-3H]NNK and [3H]NNK were used to measure pyridyloxobutylation and methylation in both globin and DNA, respectively. In globin, the level of binding increased linearly with dose. Total binding of [5-3H]NNK to globin was 3.2 to 8900 fmol/mg and total binding of [3H]NNK was 3.5 to 20,000 fmol/mg. The extents of pyridyloxobutylation of both DNA and globin were determined by measuring the amounts of 4-hydroxy-1-(3-pyridyl)-1-butanone released from each, over the dose range 15-5000 µg/kg/day. The levels of 4-hydroxy-1-(3-pyridyl)-1-butanone released were 3.2-650 fmol/mg globin, 18-3400 fmol/mg liver DNA, and 58-2180 fmol/mg lung DNA.

The extents of DNA methylation in both lung and liver were greater than pyridyloxobutylation. When the dose range was 3-5000 µg/kg/day, the levels of 7-methylguanine were 0.22-246 pmol/mmol guanine (149-167,000 fmol/mg) in liver DNA and 0.23-78 pmol/mmol guanine (160-53,000 fmol/mg) in lung DNA. In the lung, the ratio of methylation to pyridyloxobutylation decreased as the dose decreased. In contrast to globin adduct formation, DNA adduct formation did not increase linearly with dose; adduct formation was greater at lower doses than would have been predicted by extrapolation from higher doses. Thus the results of this study demonstrate that there was not a linear relationship between globin adduct formation, neither pyridyloxobutylation nor methylation, and DNA adduct formation in the liver or the lung of rats treated with NNK.

INTRODUCTION

Hemoglobin adducts have been suggested as biochemical monitors of carcinogen exposure and potentially as a measure of genotoxic risk (1, 2). The tobacco specific nitrosamine NNK3 is one of the most important carcinogens in tobacco smoke and smokeless tobacco products. This nitrosamine induces tumors of the lung, liver, nasal cavity, and pancreas in the rat (3-5). NNK is metabolically activated to reactive species that bind to hemoglobin and to DNA (5-9). The relationship between globin binding and DNA binding by NNK metabolites is the focus of this study. It is necessary to understand this relationship in order to determine what correlation exists between hemoglobin binding and DNA adduct formation in target tissues. If one could establish such a relationship, hemoglobin adduct formation could be used as a surrogate measure of DNA adduct formation.

In a previous study, globin isolated from rats treated with [5-3H]NNK was shown to be tritium labeled in a dose dependent manner. Upon mild base hydrolysis of this globin, approximately 20% of the tritium was released as HPB. This compound contains 7-mGua and O6-mGua (5, 9). Acid hydrolysis of lung and liver DNA from these animals releases HPB (7). The DNA adduct from which this compound is derived has not yet been characterized. The extent of DNA pyridyloxobutylation at different doses of NNK is unknown. The relative levels of pyridyloxobutylation and methylation of lung and liver DNA will be determined in the study presented here.

The purpose of this study was 3-fold: (a) to relate the measured levels of globin adducts to dose; (b) to relate the levels of globin adducts to DNA adducts; and (c) to investigate the relationship of methylation to pyridyloxobutylation in DNA and in globin.

MATERIALS AND METHODS

Chemicals. [5-3H]NNK (>98% pure), which contains tritium at position 5 of the pyridine ring, was used to quantify pyridyloxobutylation and release of HPB. [3H]NNK (from 2 batches, one >95% and the other >98% pure) was used to quantify methylation. They were obtained from Chemsyn Science Laboratories, Lenexa, KS, and diluted with unlabeled NNK as required (see below). HPB was synthesized, as described previously (10). O6-mGua was purchased (11), 7-mGua was purchased from Sigma Chemical Company (St. Louis, MO). Triocatinoi was obtained from Eastman Kodak Company (Rochester, NY) and redistilled before use. High purity HCl was obtained from Pierce Chemical Company (Rockford, IL).

Animal Treatments. Male F344 rats weighing 290-310 g were obtained from Charles River Breeding Laboratories, Kingston, NY. They were housed under standard conditions as described (3). Two experiments were performed. In each, groups of 3 rats were given 4 daily i.p. injections of NNK in tricatoino. This protocol was chosen for comparison to an earlier study (12). Four h after the last injection, the rats were anesthetized with halothane and sacrificed by cardiac puncture, and the liver and lung were removed for DNA isolation. Blood was collected in a 10-ml syringe containing 1 ml of 0.25% EDTA at pH 7.4. In the first experiment, groups of 3 rats were given 4 daily doses of [3H]NNK in µg/kg/day as follows (specific activity, mCi/mmol): Group 1, 1,500 (1060); Group 2, 300 (550); Group 3, 600 (270); Group 4, 1,200 (970); Group 5, 10,000 (17); or [5-3H]NNK as follows: Group 6, 150 (970); Group 7, 300 (550); Group 8, 600 (270); Group 9, 1,200 (140); Group 10, 10,000 (17). In the second experiment, groups of 3 rats were given 4 daily doses of [3H]NNK as follows: Group 1, 3 (1060); Group 2, 15 (1060); Group 3, 75 (1060); Group 4, 150 (1060); Group 5, 600 (280); Group 6, 5000 (21); or [5-3H]NNK as follows:

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2 To whom requests for reprints should be addressed.

The abbreviations used are: NNK, 4-(methyltriazeno)-1-(3-pyridyl)-1-butanone; HPB, 4-hydroxy-1-(3-pyridyl)-1-butanone; O6-mGua, O6-methylguanine; 7-mGua, 7-methylguanine; HPLC, high performance liquid chromatography.
Fig. 1. Metabolic α-hydroxylation of NNK leading to the methylation or pyridyloxobutylation of DNA or globin.

**RESULTS**

Globin Binding. Total tritium binding to globin was measured after 4 daily i.p. injections of [5-3H]NNK or [C3H1]NNK. The range of doses used was 3–10,000 µg/day. The levels of methylation of globin were 3.5–20,000 fmol/mg globin and the levels of pyridyloxobutylation 3.2–8,900 fmol/mg globin (Table 1). Methylation, which accounted for 0.06 to 0.2% of the total NNK dose, was up to 4 times higher than pyridyloxobutylation. A plot of these data as the log of dose versus the log of binding (Fig. 2) is linear (r = 0.996 for methylation and 0.997 for pyridyloxobutylation).

Previously we demonstrated that mild base hydrolysis of globin from rats treated with [5-3H]NNK released approximately 20% of the bound tritium as HPB (6). In the dose range studied here, 17–40% of the bound tritium was released as HPB. At lower doses the percentage of HPB released was greater than at higher doses. These data are presented in Fig. 2 as the log of dose versus log of HPB released; a linear relationship was observed (r = 0.995).

Globin from animals treated with [C3H1]NNK was hydrolyzed in acid for analysis of 3H-amino acids. The amount of radioactivity present in the hydrolysate after evaporation to dryness was greater than 95% of that in the globin prior to hydrolysis. Therefore less than 5% of the tritium bound to globin was present as methyl esters of glutamic or aspartic acid. The esters would have been hydrolyzed and the [3H]methanol which formed would have been lost upon evaporation of the HCl. The identities of the methylated amino acids are currently under investigation.

DNA Binding. DNA was isolated from the lung and liver of each rat. Neutral thermal hydrolysates were prepared from

<table>
<thead>
<tr>
<th>NNK dose [µg/mg]</th>
<th>Methylene</th>
<th>Pyridyloxobutylation</th>
<th>HPB released (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>[µg/kg/day]</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3 (0.015)</td>
<td>3.5 ± 1.6 (3)</td>
<td>3.7 ± 1.7 (3)</td>
<td>NDa</td>
</tr>
<tr>
<td>15 (0.073)</td>
<td>19.0 ± 4.2 (3)</td>
<td>9.4 ± 3.4 (3)</td>
<td>3.2a (1)</td>
</tr>
<tr>
<td>75 (0.360)</td>
<td>213 ± 63 (3)</td>
<td>54.9 ± 7.4 (3)</td>
<td>15.9 ± 1.3 (3)</td>
</tr>
<tr>
<td>150 (0.73)</td>
<td>318 ± 99 (6)</td>
<td>80.0 ± 30 (6)</td>
<td>30.8 ± 6.3 (6)</td>
</tr>
<tr>
<td>300 (1.45)</td>
<td>665 ± 161 (3)</td>
<td>210 ± 40 (3)</td>
<td>55.8 ± 9.6 (3)</td>
</tr>
<tr>
<td>600 (2.90)</td>
<td>1,000 ± 300 (6)</td>
<td>430 ± 19 (3)</td>
<td>174 ± 66 (6)</td>
</tr>
<tr>
<td>1,200 (5.80)</td>
<td>2,170 ± 490 (3)</td>
<td>884 ± 44 (6)</td>
<td>230 (2)</td>
</tr>
<tr>
<td>1,000 (48.4)</td>
<td>7,750 ± 840 (3)</td>
<td>3,440 ± 350 (6)</td>
<td>651 ± 164 (6)</td>
</tr>
</tbody>
</table>

- Male F344 rats (290–310 g) were given daily i.p. injections for 4 days of either [C3H1]NNK or [5-3H]NNK in trichoerin and sacrificed 4 h after the final injection. See "Materials and Methods" for details.
- Mean ± SD. Numbers of rats analyzed are shown in parentheses.
- ND, not detected; detection limit, 1.0 fmol HPB/mg globin.
- Single value (HPB was not detected in globin of the other two rats in this group).

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A chromatogram from a lung DNA hydrolysate is presented in Figure 5. The radioactivity in this peak is probably due to the incorporation of \[^{[C^{3}H_{3}]NNK}\] into the one carbon pool and then into guanine. The specific activity of guanine was less than 0.1 mCi/mmol. This is insignificant relative to the specific activity of the \[^{[C^{3}H_{3}]NNK}\] administered to the animal and would not contribute to the radioactivity detected as 7-mGua, eluting at 20 min. When a portion of the neutral thermal hydrolysate of a liver DNA sample was analyzed by HPLC using a different solvent system the amount of 7-mGua determined was the same.

The data for lung and liver DNA alkylation are summarized in Table 2. In liver, 7-mGua levels increased linearly with dose between 75 and 5000 \(\mu g/kg/day\). In lung, 7-mGua levels were similar to those observed in liver, between 3 and 600 \(\mu g/kg/day\). The curves for liver and lung in this portion of the dose range could be superimposed, as illustrated in Figure 4. However, at doses above 600 \(\mu g/kg/day\), levels of 7-mGua were lower in lung than in liver. Mild acid hydrolysates of these samples were analyzed for O6-mGua. The data are presented in Table 3. O6-mGua was not detected in the DNA from the liver or lung of animals which received less than 600 \(\mu g NNK/kg/day\). The limit of detection varied depending on the specific activity of the \[^{[C^{3}H_{3}]NNK}\] which was used for each dose. At every dose we would have been able to detect O6-mGua at less than onetenth the level of 7-mGua measured. This suggests that O6-mGua was repaired at the lower doses administered in this study.

DNA isolated from the lung and liver of animals treated with \[^{[5-'H]NNK}\] was hydrolyzed in 0.8 \(N HCl\) and analyzed for HPB released, as an indicator of pyridyloxobutylation of DNA. Previous experiments have shown that in rats treated with a single dose of 1600 \(\mu g/kg\) of \[^{[5-'H]NNK}\], approximately 50% of the radioactivity in hepatic DNA was released as HPB upon acid hydrolysis (7). Fifty \% or greater of the radioactivity bound to both lung and liver DNA was released by acid hydrolysis as HPB at the doses studied here. A representative chromatogram obtained from lung DNA of a rat treated with \[^{[5-'H]NNK}\] is illustrated in Figure 5. The major radioactive peak coelutes with HPB. In HPLC analysis of some acid hydrolysates a radioactive peak eluted at about 5 min. The presence of this peak was inconsistent and not dose dependent. Liver DNA hydrolysates contained a broad peak which eluted prior to HPB, as previously reported (7), but its presence was not consistent or dose dependent. The identities of these two peaks remain to be investigated.

The presence and amounts of HPB released from liver or lung DNA of NNK-treated rats were dose dependent and reproducible between experiments (Table 2). The DNA from the liver of one rat which received 1200 \(\mu g NNK/kg/day\) was assayed in triplicate. HPB released was 4.12 \(\pm 0.57\) (SD) pmol/\(\mu g\) guanine. This agreed well with the individual determinations on the other rats in this group. At doses of 150–600 \(\mu g/kg/day\), the levels of HPB released were similar in liver and lung (Figure 6). At doses of 75 \(\mu g/kg/day\) or less, the amount of HPB released from lung DNA was greater than that released from liver DNA (\(P < 0.05\) at 75 \(\mu g/kg/day\)). As was true for DNA methylation, when the dose was greater than 600 \(\mu g/kg/day\) the level of HPB released from liver DNA was higher than from lung DNA. At the highest dose of NNK for which DNA adduct formation was determined, 5000 \(\mu g/kg/day\), the levels of HPB released from the liver and lung DNA were not as high as would have been expected based on linear extrapolation from liver doses.

The ratio of methylation to pyridyloxobutylation decreased with decreasing dose. This effect was more pronounced in the lung than in the liver over the dose range studied.

**DISCUSSION**

The total binding of NNK to globin in rats treated with NNK was linear with dose, from 3 to 10,000 \(\mu g NNK/kg/day\). This was true for both methylation and pyridyloxobutylation of globin, although the level of methylation was always greater. The release of HPB from this globin was also linear with dose, confirming its utility as a dosimeter of NNK exposure.

In a previous study, total pyridyloxobutylation of globin was measured 24 h after a single i.p. injection of \[^{[5-'H]NNK}\] (6). The doses used were from 6 to 800 \(\mu g/kg\) and the levels of globin binding were between 5 and 750 fmol/mg. If these doses were injected on 4 consecutive days, as in the current study, one might predict globin binding would range from 20 to 3000 fmol/mg globin, yet the level of binding we measured was from 3 to 430 fmol/mg when the dose ranged from 3 to 600 \(\mu g/kg/day\). Therefore, it appears that the accumulation of \[^{[5-'H]NNK}\] binding to globin is not linear with time. This is consistent with
Table 2 7-mGua in DNA, and HPB released from DNA in F344 rats treated with NNK

<table>
<thead>
<tr>
<th>NNK dose (µg/kg/day)</th>
<th>Liver DNA (pmol/µmol guanine)</th>
<th>Lung DNA (pmol/µmol guanine)</th>
<th>HPB released</th>
<th>7-mGua/HPB ratio</th>
<th>HPB released</th>
<th>7-mGua/HPB ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>0.22</td>
<td>ND</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>15</td>
<td>0.34 ± 0.03 (3)</td>
<td>0.026 ± 0.009 (3)</td>
<td></td>
<td>13</td>
<td></td>
<td></td>
</tr>
<tr>
<td>75</td>
<td>1.4</td>
<td>0.11 ± 0.009 (3)</td>
<td></td>
<td>13</td>
<td></td>
<td></td>
</tr>
<tr>
<td>150</td>
<td>6.81 ± 2.0 (6)</td>
<td>0.37 ± 0.015 (6)</td>
<td></td>
<td>19</td>
<td></td>
<td></td>
</tr>
<tr>
<td>300</td>
<td>15.9 ± 4.8 (3)</td>
<td>0.71 ± 0.13 (3)</td>
<td></td>
<td>22</td>
<td></td>
<td></td>
</tr>
<tr>
<td>600</td>
<td>31.6 ± 7.9 (6)</td>
<td>1.3 ± 0.32 (6)</td>
<td></td>
<td>24</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1200</td>
<td>78.2 ± 8.6 (3)</td>
<td>4.4 ± 0.53 (6)</td>
<td></td>
<td>18</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5000</td>
<td>246 ± 43 (3)</td>
<td>5.0</td>
<td></td>
<td>49</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Male F344 rats (290–310 g) were given daily i.p. injections for 4 days of either [C³H³]NNK or [5-³H]NNK in trioctanoin and sacrificed 4 h after the final injection. See "Materials and Methods" for details.

See Table 3 for the specific activity of [C³H³]NNK.

Our previously reported chronic study (6).

In contrast to globin, HPB release from liver and lung DNA of NNK treated animals was not linear over the dose range studied. This may be due to saturation of the enzyme responsible for the metabolism of NNK to a pyridyloxobutylating agent in these tissues. At low doses (≤75 µg/kg/day), the levels of HPB released from lung DNA increase relative to those released from liver DNA. An explanation for this is the presence of a high affinity isozyme in this tissue which is not present in the liver. Belinsky et al. have suggested the existence of different forms of cytochrome P-450 with varying affinities for NNK (12, 16). In one study, which used a protocol identical to ours, levels of O⁶-mGua were measured in lung DNA at doses between 300 and 30,000 µg/kg/day. An immunoassay was used to detect O⁶-mGua; 3.0 pmol/µmol guanine was measured after a dose of 1000 µg/kg/day which is comparable to the levels reported here (Table 3). O⁶-mGua was reported to be linear up to a dose of 3000 µg/kg/day. Above this dose, the efficiency of alkylation, defined as the ratio of alkylation to dose, decreases. This was further investigated in a later study (16), in which
NNK was administered by s.c. injection, at doses from 30 to 30,000 μg/kg/day. After 4 days, an increase in methylation efficiency was observed at doses below 1 mg/kg/day. This is consistent with the 7-mGua levels in lung DNA reported in Fig. 4.

In the study reported here, a similar, although less significant, increase in efficiency was measured with respect to pyridyloxobutylation in both lung and liver DNA at doses less than 1200 μg/kg/day. A second potentially more relevant change in efficiency occurs at a dose of 75 μg/kg/day in lung DNA only. This is reflected in the decrease in the ratio of 7-mGua to HPB in lung DNA which occurs with decreasing dose (Table 2). One explanation for this would be the presence of different enzymes responsible for the α-hydroxylation of NNK at the methyl and methylene carbons and that in the lung the enzyme responsible for the former has a high affinity for NNK.

Despite the relative increase in the amount of HPB released from DNA at lower doses, the level of 7-mGua in DNA was always greater than the amount of HPB released from DNA. The levels of O6-mGua could not be measured in lung DNA at doses less than 600 μg/mg/kg, but based on the levels of detection (Table 3) they would be less than the levels of the HPB releasing adduct. O6-mGua is known to induce miscoding in DNA and its presence in lung DNA is likely to be involved in the initiation of lung tumors by NNK. The structure of the adduct which produces HPB upon acid hydrolysis is unknown. However, at low doses, more comparable to the level of human exposure, this adduct is quantitatively significant in lung DNA. Therefore it may play an important role in NNK tumorigenesis. This remains to be elucidated.

Independent of which of these DNA adducts is important in tumorigenesis, the purpose of this study was to determine if one could predict their levels from the levels of methylation or pyridyloxobutylation of DNA in lung DNA which occurs with decreasing dose (Table 2). One explanation for this would be the presence of different enzymes responsible for the α-hydroxylation of NNK at the methyl and methylene carbons and that in the lung the enzyme responsible for the former has a high affinity for NNK.

Fig. 8. Log log plot of HPB released from globin, after base hydrolysis, and from lung and liver DNA, after acid hydrolysis. Globin and DNA were obtained from rats treated with 5-3H]NNK (15–10,000 μg/kg/day, for 4 days).

adduct formation in globin is not linearly related to adduct formation in liver or lung DNA over the dose range studied. Methylation of liver and lung DNA, below a dose of 150 μg/kg/day, is greater than one would predict from higher doses if a linear relationship existed. In the lung, DNA methylation decreases relative to globin methylation at doses above 600 μg/kg/day. The same is true for pyridyloxobutylation of both lung and liver DNA. At lower doses, the amount of HPB released from liver DNA essentially parallels that released from globin. In contrast, the ratio of HPB released from lung DNA to that released from globin increases with decreasing dose.

NNK is a carcinogen which requires metabolic activation to bind to macromolecules. In addition, like other nitrosamines, different tissues differ in their ability to metabolize NNK. Therefore, we are interested in understanding how levels of NNK DNA adducts in the lung, a tissue with a particularly high efficiency of NNK metabolism, compare to hemoglobin adduct levels. Other studies which directly compare the levels of hemoglobin and DNA adduct formation by carcinogens which required metabolic activation are listed in Table 4. The only nitrosamines which have been studied are N-nitrosodiethy lamine and N-nitrosodimethylamine in mice (1, 17). These compounds as well as 2-acetylaminofluorene in rats (18) were found, like NNK, to bind to liver DNA to a greater extent than to globin. In rats treated with urethan, trans-4-dimethylaminostilbene, or fluoranthene the levels of globin alkylation were greater than those in DNA (19–21). Approximately equal arylation of hemoglobin and liver DNA occurred in mice treated with benzo(a)pyrene or 7-bromomethylbenz(a)anthracene (22, 23).

Of these compounds, only for 2-acetylaminofluorene and trans-4-dimethylaminostilbene was the correlation between hemoglobin and DNA alkylation measured over a large dose range, 0.1 to 100 and 0.005 to 100 μmol/kg body weight, respectively. For both of these aromatic amines, dose and adduct formation in liver DNA and hemoglobin were linearly related, unlike NNK. The amount of information available comparing hemoglobin binding to DNA binding in extrahepatic tissues is quite limited (Table 4). The level of 7-mGua in kidney DNA is greater than the methylation of hemoglobin in rats treated with a single relatively high dose of N-nitrosodimethylamine. As with the other carcinogens studied, the level of
DNA adducts in the liver are greater than that in the DNA of extrahepatic tissue. This is not the case for NNK methylation or pyridyloxobutylation of lung DNA at doses less than 600 μg/kg/day. The relationship between globin and lung DNA adduct formation by NNK is relevant because NNK is a lung carcinogen and may be important in human cancers associated with tobacco use.

The release of HPB from the globin of snuff and cigarette users is reported in Paper 1 (25). In order to understand how this reflects what occurs in the DNA of these individuals one must understand the mechanism of globin adduct formation. The question is: do changes in globin adducts reflect changes in metabolism in the target tissue? That is, can one use the levels of HPB released from hemoglobin of tobacco users as a measure of the ability of these individuals to activate NNH and NNK to DNA binding species? To answer this question one must know where the globin adduct is formed. Is the reactive species formed in the liver and transported into the RBC where it binds to globin? The transport of a reactive species into RBC was demonstrated in vitro for N-nitrosodimethylamine (26). In this study, we have established the relationship between NNK, as the exposure level is lowered, the extent of metabolism in the lung increases relative to what occurs in the liver. Does this contribute to globin adduct formation? In part this depends on the nature of the reactive species which is transported and whether or not the RBC itself is involved in further metabolism of this compound.

In this study, we have established the relationship between the dose of NNK, a carcinogen requiring metabolic activation, and the level of globin adducts as well as the relationship between globin adducts and DNA adducts in the lung and liver, over a wide dose range. The first relationship is a linear one, but the second is not. In the rat, NNK is primarily a lung carcinogen at low dose. In humans, NNK is thought to be a causative agent for tobacco related lung cancers. Understanding the significance of NNK globin adducts to the risk of lung cancer in humans requires further mechanistic studies on the relationship of these adducts to DNA adducts in the target tissue.

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