LACK OF COMBINATION HEPATOCARCINOGENICITY OF HARMAN, NORHARMAN AND AMITROLE WHEN GIVEN WITH NaNO₂ IN THE RAT

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ABSTRACT — N-Nitrosocompounds, which induce cancers in various organs, may be formed endogenously with intake of amino compounds such as secondary amines and sodium nitrite (NaNO₂) in combination. The present study was performed to investigate whether three amino compounds, 1-methyl-9H-pyrido[3,4-b]indole (harman), 9H-pyrido[3,4-b]indole (norharman) and 2-amino-1,3,4-triazole (amitrole), might be converted in vivo to compounds capable of promoting hepatocarcinogenesis when given with NaNO₂. However, in an 8-week model, no modifying potential was evident in terms of numbers and areas of putative preneoplastic glutathione S-transferase placental form (GST-P)-positive foci in any of the groups receiving paired treatments. These results demonstrate that combinations of harman, norharman and amitrole with NaNO₂ lack promoting effects for liver carcinogenesis in our medium-term bioassay system.

KEY WORDS: Hepatocarcinogenesis, Harman, Norharman, Amitrole, NaNO₂

INTRODUCTION

N-Nitroso carcinogens may be formed endogenously on intake of amino compounds such as secondary amines and sodium nitrite (NaNO₂) in combination (Mirvish, 1975). In the present study we investigated whether this might also be the case with 1-methyl-9H-pyrido[3,4-b]indole (harman) and 9H-pyrido[3,4-b]indole (norharman), two β-carboline derivatives found in some vegetables and in pyrolysis products of tobacco and meat (Poindexter and Carpenter 1962; Takase and Murakami, 1966; Takeuchi et al., 1973; Gross, 1990). Both compounds do demonstrate mutagenic activity when in combination with other nonmutagens, such as aniline and o-toluidine (Nagao et al., 1977). It is likely that humans are exposed to these compounds in daily life and both chemicals, while lacking mutagenicity themselves, possess co-mutagenic potential in several in vitro assays (Umezawa et al., 1978; Nagao et al., 1977; Totsuka et al., 1998). Another compound of interest is 2-amino-1,3,4-triazole (amitrole), a pesticide whose use is now limited to non-crop applications because of its induction of thyroid tumors in mice and rats by a non-genotoxic mechanism which involves interference with the functioning of thyroid peroxidase (IARC, 2001). N-Nitroso compounds are known to induce tumors in various organs, and the rat liver is a common target of nitrosamines (Lijinsky, 1987). Therefore, investigation of carcinogenicity of NaNO₂, in combination with amino compounds (harman, norharman and amitrole) is of interest and importance.

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Our medium-term bioassay using the rat liver is well established to have utility for accurate prediction of hepatocarcinogenic activity (Ito et al., 1988; Ito et al., 2003; Pitot and Dragan, 2001; Shirai et al., 1999), allowing rapid screening of test substances using putative preneoplastic glutathione S-transferase placental form (GST-P)-positive foci as surrogate end point marker lesions. Testing of hepatocarcinogens at various doses in the model has revealed quantitative values for GST-P-positive foci to be dose-dependent, in line with data for incidences of hepatocellular carcinomas (Ogiso et al., 1985; Ogiso et al., 1990; Tatematsu et al., 1988).

The present study was performed to determine whether typical amino compounds (harman, norharman and amitrole) individually fed with NaNO₂ might have modifying potential on liver carcinogenesis using our rat medium-term liver carcinogenesis bioassay system.

MATERIALS AND METHODS

Animals
Male F344/DuCrj rats were obtained at 5 weeks of age from Charles River Japan (Atsugi, Kanagawa, Japan), housed two or three in a plastic cage with hard wood chips for bedding, and fed a powdered diet MF (Oriental Yeast, Co., Ltd., Tokyo, Japan) and water ad libitum. The animals were kept in an environmentally controlled room maintained at a temperature of 22 ± 3°C, a relative humidity of 55 ± 10% and with 12-hr light/dark cycle. They were used in this study after a one-week acclimation period.

Chemicals
Diethylnitrosamine (DEN) was obtained from Tokyo Kasei Kogyo Co., Ltd. (Tokyo, Japan). Amitrole standard (Cat No. 016-14011) and sodium nitrite were purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan) and harman (Cat No. H-7258) and norharman (Cat No. N-6252) from Sigma-Aldrich (St. Louis, MO).

Experimental design
The experimental design is shown in Fig. 1. A total of 90 rats were divided into 6 groups, all receiving a single intraperitoneal (i.p.) injection of DEN (200 mg/kg body weight) dissolved in saline to initiate hepatocarcinogenesis. After 2 weeks, the rats in group 1 and 6 were then administered basal diet and diet containing amitrole (0.01%), respectively. Animals in groups 2, 3, 4 and 5 received a diet containing basal diet, harman (0.05%), norharman (0.05%) and amitrole (0.01%), each in combination with 0.1% NaNO₂ (given in the drinking water) for the remaining experimental duration. These (harman, norharman, amitrole) doses were chosen based on the results of previous studies (Hagiwara et al., 1980, 1990, 1992; Steinhoff et al., 1983). All rats were subjected to two-thirds partial hepatectomy at week 3. Throughout the experiment, the animals had free access to food and water, and body weights were measured once a week. Food and water consumption were also recorded once a week. Surviving rats in each group were killed for examination at week 8. At autopsy, livers were excised and 3 mm thick slices were cut with a razor blade and fixed in 10% buffered formalin for immunohistochemical examination of GST-P-positive foci.

Immunohistochemical staining and measurement of GST-P-positive foci
The avidin-biotin-peroxidase complex (ABC) method was used to stain GST-P-positive foci. After deparaffinization, liver sections were treated sequentially with normal goat serum, anti-rabbit GST-P antibody (MBL Co., Ltd., Nagoya, Japan; 1:2000), and...
Combination effects of amino compounds with NaNO2.

Biotin-labeled goat anti-rabbit IgG (1:200) for 1 hr followed by ABC. The sites of peroxidase binding were visualized by the diaminobenzidine method and the nuclei were counter-stained with hematoxylin. The numbers and the areas of GST-P-positive foci >0.2 mm in diameter and the total areas of the liver sections examined were measured using a color video image processor (IPAP, Sumika Technos Corp., Osaka, Japan).

Statistical analysis

The significances of intergroup differences in numerical data obtained for body weights, organ weights and GST-P-positive foci were assessed using the two-sided Student’s t-test (Gad and Weil, 1982). For the incidences of histopathological lesions, the significance of differences observed between the control and treated groups was evaluated with Fisher’s exact probability test (Fisher, 1955; Gart et al., 1979). The levels of significance were set at p<0.05 and 0.01.

RESULTS

Significant retardation of body weight gain was observed in rats treated with amitrole (Fig. 2), along with a tendency for decrease in food and water consumption values (data not shown). A tendency for increase in water consumption was observed for rats treated with harman (data not shown). The final body weights and relative organ weights are summarized in Table 1. The final body, liver and kidney weights were significantly decreased in the amitrole-treated groups.

![Graph showing body weight changes in rats.](image)

Table 1. Final body weight and organ weight data for rats.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>No. of rats</th>
<th>Final body weight</th>
<th>Liver</th>
<th>Kidneys</th>
<th>Thyroids</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>15</td>
<td>269.6 ± 12.4</td>
<td>7.96 ± 0.32</td>
<td>1.80 ± 0.09</td>
<td>0.017 ± 0.002</td>
</tr>
<tr>
<td>NaNO2 alone</td>
<td>15</td>
<td>266.9 ± 11.3</td>
<td>7.97 ± 0.39</td>
<td>1.82 ± 0.08</td>
<td>0.017 ± 0.002</td>
</tr>
<tr>
<td>NaNO2 + Harman</td>
<td>14</td>
<td>262.3 ± 9.1</td>
<td>7.90 ± 0.47</td>
<td>1.80 ± 0.06</td>
<td>0.018 ± 0.003</td>
</tr>
<tr>
<td>NaNO2 + Norharman</td>
<td>15</td>
<td>265.1 ± 10.8</td>
<td>8.11 ± 0.36</td>
<td>1.78 ± 0.11</td>
<td>0.017 ± 0.002</td>
</tr>
<tr>
<td>NaNO2 + Amitrole</td>
<td>14</td>
<td>212.3 ± 22.5**</td>
<td>5.43 ± 0.85**</td>
<td>1.28 ± 0.12**</td>
<td>0.139 ± 0.027**</td>
</tr>
<tr>
<td>Amitrole alone</td>
<td>13</td>
<td>219.7 ± 10.3**</td>
<td>5.94 ± 0.73**</td>
<td>1.32 ± 0.10**</td>
<td>0.159 ± 0.021**</td>
</tr>
</tbody>
</table>

**: Significantly different from control group at p<0.01.
while thyroid weights were increased.

Data for the numbers and the areas of GST-P-positive foci in the liver are summarized in Fig. 3. With harman, norharman and amitrole in combination with NaNO₂ they were not significantly altered from the values for GST-P-positive foci in the liver treated with NaNO₂ alone. Similarly, amitrole-alone exerted no modification potential. Histopathological findings of the thyroid and kidney are summarized in Table 2. In the thyroids, diffuse follicular cell hyperplasia was observed in all rats exposed to amitrole. Crystal deposition was observed in the kidneys of animals receiving harman, and inflammation of tubules with exposure to norharman.

**DISCUSSION**

It has been reported that secondary amines react with nitrite under acidic conditions to form nitrosamines (Kawanishi et al., 1981; Kuenzing et al., 1984). Aminopyrine is one such amino compound, reacting with NaNO₂ to form dimethylnitrosamine, a potent liver carcinogen (Lijinsky et al., 1973; Lijinsky and Greenblatt, 1972; Scheunig et al., 1979), thereby

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**Table 2.** Histopathological findings for the thyroids and kidneys.

<table>
<thead>
<tr>
<th>Organ</th>
<th>NaNO₂</th>
<th>Test chemical</th>
<th>Harman</th>
<th>Norharman</th>
<th>Amitrole</th>
<th>Amitrole</th>
</tr>
</thead>
<tbody>
<tr>
<td>and Findings</td>
<td>Level (%)</td>
<td>------</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>No. of animals examined</td>
<td>(15)</td>
<td>(15)</td>
<td>(14)</td>
<td>(15)</td>
<td>(14)</td>
<td>(13)</td>
</tr>
<tr>
<td>Thyroids</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal</td>
<td>15</td>
<td>15</td>
<td>14</td>
<td>15</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Diffuse follicular cell hyperplasia</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>14</td>
<td>13</td>
</tr>
<tr>
<td>Kidneys</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal</td>
<td>15</td>
<td>15</td>
<td>9</td>
<td>12</td>
<td>14</td>
<td>13</td>
</tr>
<tr>
<td>Crystal deposition</td>
<td>0</td>
<td>0</td>
<td>11</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Tubulitis</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>2</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Mineralization</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Dilatation, renal tubule</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

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**Fig. 3.** Quantitative data for GST-P-positive foci in the liver of rats.
causing a significant increase in GST-P-positive foci and induction of hepatocellular adenomas in a rat multi-organ carcinogenesis model (Yada et al., 2002). In the present study, however, GST-P-positive foci in the liver were not increased by the combined treatment of any of the amino compounds (harman, norharman or amitrole) with NaNO2.

It has been reported that mutagenic compounds are formed from norharman and aniline in vitro (Umezawa et al., 1978; Nagao et al., 1977; Totsuka et al., 1998). Moreover, GST-P-positive foci in the liver clearly developed in rats fed 9-[(4’-aminophenyl)-9H-pyrido[3,4-b]indole[aminophenyl]norharman], produced by their interaction (Totsuka et al., 2004). However, Hagiwara et al. (1980) found that oral administration of norharman with aniline failed to demonstrate any carcinogenicity in rats (Hagiwara et al., 1980). It is probable that the lack of promotion effects of combined treatment with amino compounds (harman, norharman or amitrole) and NaNO2 in the liver may be because they do not effectively interact to produce mutagenic or carcinogenic compounds in vivo. Further investigations will be required to elucidate this point with in vitro mutagenicity tests and/or in vivo initiation assay.

Crystal deposition or tubulitis in the kidney were evident in the present study with harman or norharman treatment, in line with earlier findings of severe toxic renal lesions with these compounds at 1000 ppm, and to a lesser extent with 200 ppm (Hagiwara et al., 1990).

The fact that amitrole lacked modifying potential for induction of GST-P-positive foci in this study is also in agreement with the report of no induction of liver tumors, despite multiple thyroid lesions with long-term oral treatment in rats (Steinhoff et al., 1983). In the present study, diffuse follicular cell hyperplasia in the thyroids was marked in rats given amitrole, again in line with previous results (Wynford-Thomas, 1982).

In conclusion, the present study demonstrated that combinations of harman, norharman and amitrole with NaNO2 lack promoting potential for liver carcinogenesis in our medium-term bioassay system. Whether promoting effects might be exerted in organs other than the liver remains to be clarified.

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