Carcinogenesis by Derivatives of 1-Nitroso-3,5-dimethylpiperazine in Rats

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ABSTRACT

Four mononitrosopiperazines were administered to groups of 20 female Fischer 344 rats to compare their effectiveness as carcinogens. The four, 1-nitroso-3,5-dimethylpiperazine, its 4-acetyl derivative, its 4-benzoyl derivative, and 1-nitroso-3,4,5-trimethylpiperazine, were given as 0.7% solutions in drinking water, 100 ml to each rat per week. The length of treatment varied from 26 weeks for nitrosotrimeylpiperazine to 50 weeks for 1-nitroso-3,5-dimethyl-4-benzoylpiperazine. Dimethyl- and trimethyl nitrosopiperazine gave rise to virtually 100% incidence of undifferentiated lymphomas of the thymus and leukemias within 30 weeks (in contrast to the non-C-methylated analogs which are noncarcinogenic or only weakly so). Acetyldimethyl nitrosopiperazine was also a potent carcinogen, all of the rats treated with it dying within 30 weeks with tumors of the esophagus. In contrast, benzoyldimethyl nitrosopiperazine was weakly carcinogenic, inducing only a small number of tumors of the forestomach and reducing the normal life span of the rats very little.

INTRODUCTION

It has been known for some time that, while 1,4-dinitrosopiperazine is a quite potent carcinogen in rats [giving rise to tumors of the liver and esophagus (2, 10)], mononitrosopiperazine and 1-methyl-4-nitrosopiperazine are either very weak carcinogens or not carcinogenic (6, 11). This parallels in general the mutagenic activity of the 3 compounds in bacteria (3, 15). It seemed possible that the reason for the lack of activity by the mononitrosopiperazines might be their basic character, which might prevent the entry of these molecules in their ionized form into cells.

The finding that the 2,6-dimethyl derivative of dinitrosopiperazine, which was considerably more potent as a carcinogen than was the parent compound (10), loses the nitroso group at position 1 relatively readily (18) in acidic solution led us to an interest in examining the carcinogenicity of the product, 1-nitroso-3,5-dimethylpiperazine, which is also a base. When, contrary to expectation, this compound proved to be a very potent carcinogen in rats, it was decided to examine the carcinogenicity of 1-nitroso-3,4,5-trimethylpiperazine and 2 acylated derivatives, 4-acetyl- and 4-benzoyl-1-nitroso-3,5-dimethylpiperazine (Chart 1).

All of these compounds were administered to female F344 rats as equimolar solutions in drinking water, in which they were all sufficiently soluble, for a fixed period, after which the animals were kept until death with tumors. The concentrations of the compounds were equivalent to 120 mg of dinitroso-2,6-dimethylpiperazine per liter of drinking water, which had previously been administered to the same strain of rat.

MATERIALS AND METHODS

Chemicals. 1-Nitroso-3,5-dimethylpiperazine was prepared as described previously (18) and was characterized as cis-diequatorial by its NMR spectrum. NMR (CDCl3) δ 1.13 (d, 3H, syn-3-Me, J = 6.25 Hz); δ 1.21 (d, 3H, anti-5-Me, J = 6.02); δ 2.13 (ddd, 1H, syn-2ax-H, Jgem = -12.87, J2a-3 = 11.03, J2ax-6axS1); δ -2.7 (m, 1H, syn-3H); δ 3.08 (m, 1H, anti-5H); δ 3.27 (ddd, 1H, anti-6a-H, Jgem = -11.72, J6a-5 = 11.41, J6a-2e = 1); δ 4.63 (ddd, 1H, anti-6eq-H, Jgem = -11.72, J6e-5a = 2.56, J6e-5a = 1.5); δ 4.97 (ddd, 1H, syn-2eq-H, Jgem = -12.87, J2a-3 = 3.16, J2a-6a = 1.5).

1-Nitroso-3,4,5-trimethylpiperazine was prepared by dissolving 7 g (0.05 mol) of 1-nitroso-3,5-dimethylpiperazine in a mixture of 90% formic acid (7.5 ml, 0.15 mol) and formalin (formaldehyde solution; 12.2 ml, 0.15 mol) and boiling under reflux for 24 hr (12). The mixture was poured onto ice, basified (NaOH), and extracted with CHCl3 (4 × 100 ml). The CHCl3 extracts were dried (MgSO4) and evaporated in a vacuum to give an orange oil (7.5 g, 96%) which was distilled to give a yellow oil, b.p. 76–79°C/0.2 mm mercury IR (liquid film) 2965 (C=H); 2780 (N=CH3); 1450, 1045 (N=NO). MS m/z (%): 127 (40, M+ - 30); 113 (80); 70 (80); 42 (100). NMR (CDCl3) δ 1.16 (d, 3H, syn-3-Me, J = 6.02 Hz); δ 1.24 (d, 3H, anti-5-Me, J = 6.23); δ 2.29 (s, 3H, N-CH3); δ 3.50 (ddd, 1H, anti-6a-H, Jgem = -12.96, J6a-5 = 10.98, J6a-2e = 1.07); δ 4.58 (ddd, 1H, anti-6eq-H, Jgem = -12.96, J6e-5a = 3.36, J6e-2e = 

1 This work was supported by Contract N01-CO-75380 with the National Cancer Institute, NIH, Bethesda, Md. 20205.
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Received August 12, 1980; accepted November 21, 1980.
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Animal Treatments. Each of 4 dimethylnitrosopiperazines was administered in aqueous solution to a group of 20 female F344 rats of the colony of the Frederick Cancer Research Center which were bred and maintained under specific-pathogen-free conditions. The animals were 8 to 9 weeks old at the beginning of the experiments. The rats were housed in groups of 4 in plastic cages with wire mesh bottom. They were fed Wayne Lab-Blox rat diet ad libitum. Each cage of 4 animals was given 80 ml of nitrosamine solution on 5 days of each week; almost all of the solutions were consumed with little spillage. On the remaining 2 days of each week, tap water was given so that the rats could make up any water deficit they had incurred. Treatment continued for 50 weeks or until animals started to die; the length of treatment and the concentrations of the 4 nitrosamines used are given in Table 1. A group of 20 untreated female F344 rats was a contemporary control of the benzoyl compound, but the animals treated with the remaining 3 compounds died long before any of their untreated controls. The animals were allowed to die naturally except for a few which were killed when moribund. Each animal was given complete necropsy, and all lesions and major tissues and organs were fixed for histological examination.

RESULTS

The pattern of death of the treated rats and of the untreated controls is given in Table 1. While survival of the animals given the N-benzoyl compound was very similar to that of untreated rats, survival of the rats given the other 3 nitrosamines was very poor, and few survived beyond the 35th week after the beginning of treatment. Almost all of the rats in these 3 groups died with induced undifferentiated lymphomas of the thymus and leukemia (nitrosodimethylpiperazine and nitroso-3,4,5-trimethylpiperazine), or neoplasms of the esophagus (nitroso-4-acetyldimethylpiperazine). On the other hand, most of the animals treated with nitroso-4-benzoyldimethylpiperazine died much later and, except for tumors of the forestomach and liver, had a spectrum of tumors similar to that of untreated rats. The tumors found in the treated rats and in the controls are listed in Table 2. The tumors were characterized as follows.

Undifferentiated Lymphoma of Thymus with or without Leukemia. The thymus was markedly enlarged. The cortical portion or the entire thymic architecture was replaced by a diffuse cell pattern made up of relatively large cells containing large round to oval nuclei with distinct nucleoli and scanty cytoplasm (Figs. 1 and 2). Scattered among these cells were large phagocytic histiocytes with cytoplasm containing scattered cell debris. Capsular invasion was observed.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Concentration in water (mg/liter)</th>
<th>Duration of treatment (wk)</th>
<th>Total dose (mg)</th>
<th>No. of animals at following wk</th>
<th>No. of tumor-bearing animals</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nitroso-3,5-dimethylpiperazine</td>
<td>100</td>
<td>29</td>
<td>290 (2.0)</td>
<td>20</td>
<td>20</td>
</tr>
<tr>
<td>Nitroso-3,4,5-trimethylpiperazine</td>
<td>110</td>
<td>26</td>
<td>286 (1.8)</td>
<td>20</td>
<td>20</td>
</tr>
<tr>
<td>Nitroso-3,5-dimethyl-4-acetyl-</td>
<td>130</td>
<td>30</td>
<td>390 (2.1)</td>
<td>20</td>
<td>20</td>
</tr>
<tr>
<td>Nitroso-3,5-dimethyl-4-benzoyl-</td>
<td>172</td>
<td>50</td>
<td>860 (3.5)</td>
<td>20</td>
<td>20</td>
</tr>
<tr>
<td>piperazine</td>
<td>Un treated</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Numbers in parentheses, mmol.

b Killed at 132 weeks.
Cells similar to those in the thymus generally were also present in the spleen, liver, bone marrow, and other organs in rats with leukemia.

**Olfactory Carcinoma.** The tumor consisted of densely packed cells with round or irregular oval nuclei, ill-defined borders, and scanty eosinophilic cytoplasm (Fig. 3). Tumor cells grew around or along the vessels. Rosette and pseudorosette formation was prominent. Mitotic figures were occasionally seen. The neoplasms were present in the upper portion of the nasal cavity and often invaded bone and brain.

**Nasal Carcinomas.** Nasal carcinomas which were basal or squamous cell carcinomas were seen in the anterior portion of the nasal cavity.

**Carcinomas of the Esophagus.** Carcinomas of the esophagus were either basal cell or a mixture of basal and squamous cells and often contained keratin. Carcinomas in the group treated with nitrosoacetyldimethylpiperazine were large and invasive. Squamous metaplasia of the trachea and bronchus were present in 6 rats, and a squamous cell papilloma was seen in one rat. Eleven of the rats with carcinomas of the esophagus and squamous metaplasia of the trachea and bronchus developed purulent bronchitis and abscesses of the lung. In some rats, there was aspiration of keratin or food particles.

**Carcinomas of the Tongue and Forestomach.** These carcinomas were basal cell carcinomas, often with keratin.

**Lesions Other than Neoplasms.** One rat treated with nitrosodimethylpiperazine had severe hepatic necrosis, and another had hepatic vein thrombosis, an unusual lesion. One rat given nitrosotrimethylpiperazine had acute bacterial endocarditis, also a rare lesion.

**DISCUSSION**

The induction of undifferentiated lymphomas of the thymus and leukemias by the 2 unacylated nitrosodimethylpiperazines is surprising, since tumors of this type have heretofore not been reported as induced by nitrosamines [although some nitrosamides have induced leukemias and lymphomas in mice (4) and in rats (2)]. The mechanism of induction of these tumors is not known, although alkylation of nucleic acids is suspected in the case of nitrosoalkylureas (5). It is difficult to imagine a mechanism of this type for the 2 mononitrosopiperazines examined here, particularly in view of the very low, almost negligible mutagenic activity of the compounds in the microsomal activated bacterial mutagenicity test. In addition, the analogous compound dinitrosopiperazine was not found to alkylate DNA of the target organ, the liver, of rats to a detectable extent in vivo (9).

Although dinitroso-2,6-dimethylpiperazine loses the nitroso group at position 1 with ease to form 1-nitroso-3,5-dimethylpiperazine (18), no tumors of the thymus were observed in Fischer rats treated with the former compound, suggesting that any denitrosation in vivo occurred at too low a rate to be effective in thymic tumor induction.

Acylation of the 4-position of 1-nitroso-3,5-dimethylpiperazine profoundly changed the carcinogenic activity of the molecule. Acetylation did not change the effectiveness of the compound, since rats treated with the 4-acetyl derivative died within 30 weeks, but the organ in which tumors were induced

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4 W. A. Andrews and W. Lijinsky, unpublished information.
was the esophagus instead of the thymus. Indeed, no tumors of the thymus were seen in these animals, suggesting that little, if any, hydrolysis of the amide occurred in vivo. Benzoylation at position 4, on the other hand, produced in 1-nitroso-3,5-dimethyl-4-benzoylpiperazine a very weak carcinogen which induced only a few forestomach papillomas and had little effect on position 4, on the other hand, produced in 1-nitroso-3,5-dimethyl-4-benzoylpiperazine a very weak carcinogen which induced only a few forestomach papillomas and had little effect on the carcinogens had low liposolubility, and some of the non-carcinogens were free bases. 1-Nitroso-4-benzoyl-3,5-dimethylpiperazine had high liposolubility (partition coefficient, P = 35) in comparison with the carcinogenic methylated dinitrosopiperazines (P = 0.3 to 1.5). The orientation of the methyl groups in the nitrosodimethylpiperazines tested is possibly a determining factor in their organ specificity. The 2 compounds that induce the thymic lymphomas, nitroso-3,5-dimethyl- and nitroso-3,4,5-trimethylpiperazine, have the C-methyl groups cis-diequatorial. This is apparent from the methyl coupling constants (JCH3-H = 6 Hz) (7) and from the large vicinal methine-axial-H coupling (JChs-H = 11 to 11.4 Hz) which indicates 2 trans-diaxial protons and, therefore, equatorial methyl groups. Conversely, the esophageal carcinogens, nitroso-4-acetyl-3,5-dimethyl- and 1,4-dinitroso-2,6-dimethylpiperazine (10), as well as nitroso-4-benzoyl-3,5-dimethylpiperazine, have cis-diaxial methyl groups. The methyl coupling constant is larger (JCH3-H = 7 Hz) and there is a trans-equatorial-axial vicinal coupling constant (JChs-H = 5.5 Hz). Axial methyl groups alpha to a nitroso group (1) or an amide function (8) are the usual observation. The thymic lymphoma has been transplantable to other F344 rats.9 The olfactory carcinomas are suggestive of neoplasms described as esthesioneuroepithelioma (14). Further studies are under way to determine the cell type of both the thymic lymphomas and the olfactory carcinoma (13).

REFERENCES

Fig. 1. Undifferentiated lymphoma of the thymus. Thymus is replaced diffusely by relatively large cells. H & E, × 160.

Fig. 2. Undifferentiated lymphoma of the thymus. Cells have large, round nuclei with distinct nucleoli and scanty cytoplasm. H & E, × 160.

Fig. 3. Carcinoma of the nasal cavity. There are densely packed cells with round or irregular nuclei and little cytoplasm growing in islands. Cells are lined up in columns at the periphery. H & E, × 400.
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