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Carcinogenicity of Methylated Dinitrosopiperazines in Rats[1]

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SUMMARY

Dinitrosopiperazine and four of its homologs were tested as carcinogens by feeding to rats at equimolar doses in drinking water. All 5 compounds induced tumors of the olfactory epithelium and/or esophagus in almost 100% of the animals, the tumors being the cause of death. The comparison of carcinogenic potency was based on the time to death of the animals after initiation of treatment by the different compounds.

One homolog, 2,5-dimethyldinitrosopiperazine, was approximately as potent as was dinitrosopiperazine. Dinitrosohomopiperazine, 2-methyldinitrosopiperazine, and 2,6-dimethyldinitrosopiperazine were considerably more effective carcinogens than was dinitrosopiperazine, a smaller dose killing the animals in a shorter time. The 2,6-dimethyl derivative was the most effective compound. The increased carcinogenicity of the homologs might be related to steric effects or to activation at the α carbon atoms to the *N*-nitroso group.

INTRODUCTION

To investigate the effects of small changes in chemical structure on carcinogenic activity and to aid in interpreting them in terms of the mechanism of action of this type of carcinogen, a series of methylated derivatives of nitrosopiperazine was prepared and tested in rats. While dinitrosopiperazine has been tested and found to be a potent carcinogen in rats (1, 3) and in mice (7), 1-nitrosopiperazine (2) and *N*-methylnitrosopiperazine (1) appear to be very weakly carcinogenic, perhaps because they are both bases. On the other hand, none of the compounds we include here appears to have been tested previously, including dinitrosohomopiperazine, the next higher homolog of dinitrosopiperazine (Chart 1).

To enable comparisons of carcinogenic potency of the compounds, it was planned to administer all of the homologs to the rats in drinking water at doses equal in molarity to the standard dose of 100 mg of dinitrosopiperazine per liter and for the same length of time. As it turned out, it was not possible to continue the treatment with several of the compounds for the full course of 50 weeks, as was done with dinitrosopiperazine, because in those cases the compounds were so potent that several animals died as early as 30 weeks after the beginning of the experiment, and treatment was stopped.

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MATERIALS AND METHODS

Chemicals. All of the dinitrosopiperazines were prepared from the amines (which were obtained in pure state from Aldrich Chemical Co., Milwaukee, Wis.) by reaction with sodium nitrite (3 moles/mole of amine) in dilute (30%) acetic acid (4 moles of acid per mole of amine). The acetic acid solution of the amine was cooled in ice, and the solid sodium nitrite was added slowly with shaking. The nitroso compound started to appear within minutes as a yellow precipitate or an oil. After 1 hr the flask was removed from the ice bath, and the reaction was completed at room temperature for a further 2 hr. Then solid potassium hydroxide was added, with cooling, until the solution was alkaline, and the solution was extracted twice with an equal volume of methylene chloride. The extract was shaken twice with 20 ml of 5 N HCl, and the methylene chloride layer was evaporated at room temperature in a stream of nitrogen to remove the solvent. The residual nitrosopiperazine crystallized on standing at room temperature or in the refrigerator. Each of the solid dinitrosopiperazines was purified by crystallization of small portions from hot water. On cooling, the nitrosamine crystallized easily and, after filtration, the mother liquor was used to dissolve a further portion of nitrosamine. In this way large quantities of nitrosopiperazines could be purified without using huge volumes of water and thereby losing appreciable amounts of compound. Each nitrosopiperazine was identified empirically by its mass spectrum (although the 2 dimethyl derivatives could not be distinguished in this way), each giving a prominent molecular ion and the expected fragmentations, without detectable impurities. Further assurance of purity was given by sharpness of melting point and UV absorptivity. The yields and some physical characteristics of the dinitrosopiperazines are given in Table 1.

Animal Treatments. Each nitrosopiperazine was given to a group of 15 male and 15 female Sprague-Dawley rats, 8 to 10 weeks old, bred and maintained in plastic cages in a closed colony of this laboratory and fed Purina laboratory chow *ad libitum*. Dinitrosopiperazine was given as drinking water solution, containing 100 mg/liter (0.01%), by dissolving 0.4 g in hot water and diluting this to 4 liters with distilled water (pH 6.8 to 7). Feeding solutions of the other compounds at the same molar concentration were prepared

similarly. Each cage of 3 rats was given 60 ml of solution each day, 5 days/week. After 2 or 3 weeks of adjustment, almost all of the solution was consumed overnight by each cage of animals. The remaining 2 days of each week the animals were given tap water *ad libitum*. The stock feeding solutions were kept in the dark and there was no detectable decomposition (as determined by measurement of UV absorbance of the solutions) even when kept several weeks. Treatment with 2 of the compounds (dinitrosopiperazine and the 2,5-dimethyl derivative) continued for 50 weeks, but with the other 3 compounds (2-methyldinitrosopiperazine, 2,6-dimethyldinitrosopiperazine, and dinitrosohomopiperazine) treatment was stopped at 31 and 33 weeks because several animals in those groups had died with tumors.

Animals were allowed to die naturally or were killed when moribund. They were submitted to complete necropsy, and all organs with tumors or other lesions were fixed for histological examination.

RESULTS

The concentration of each nitrosopiperazine in the water given to the rats is shown in Table 2, together with the length of treatment and the total dose of compound administered. The females consumed the same dose as did the males but, being considerably smaller, received more than twice the dose per unit body weight as did the males. Nevertheless, as seen in the survival figures of Table 2, there was no significant difference in rate of death from tumors between males and females in most groups; there did seem to be such a difference in the group treated with 2,6-dimethyldinitrosopiperazine and, perhaps, in the group given 2,5-dimethyldinitrosopiperazine.

In all groups the tumors induced by the treatment with the nitrosamine appeared to be the cause of death. The organ distribution of the tumors and their numbers were similar in all of the groups, so that time of death of the animals became an index of the potency of the administered compound as a carcinogen. By this criterion the parent compound, dinitrosopiperazine, was the least potent carcinogen among them, with its 2,5-dimethyl derivative a little more potent. On the other hand, dinitrosohomopiperazine, 2-methyldinitrosopiperazine, and 2,6-dimethyldinitrosopiperazine were considerably more effective than dinitrosopiperazine, smaller doses of the homologs leading to death from tumors much more quickly than doses of the parent compound. The numbers and distribution of tumors are shown in Table 3.

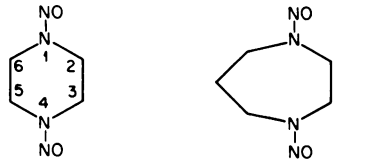

Chart 1. Chemical structures of dinitrosopiperazine and dinitrosohomopiperazine.

Tumors were present in the nasal turbinates, upper gastrointestinal tracts (tongue, oropharynx, esophagus, and nonglandular stomach), livers, and brains. In the nasal turbinates, tumors were of epithelial origin and appeared to originate from metaplastic foci of the mucous membranes. Differentiation into keratinizing squamous cell carcinomas, acinar-forming adenocarcinomas, and mixtures of both were seen. These carcinomas commonly invaded the ethmoturbinates, cribriform plates, and olfactory lobes, to cause death by displacing and destroying the brain. Adenocarcinomas closely resembled those induced by di-*n*-propylnitrosamine (6) and diethylnitrosamine (4) in hamsters.

Tumors of the upper gastrointestinal tract were squamous papillomas and squamous cell carcinomas, present in about equal proportions in the affected groups. Liver tumors occurred only in the dinitrosopiperazine-treated group and were both hepatomas and hepatocellular carcinomas. The 1st tumor was found in an animal dying at the 48th week of the experiment. Three tumors in the experiment originated in the brain: 1 tumor each in the 2,5-dimethyldinitrosopiperazine, dinitrosohomopiperazine, and dinitrosopiperazine groups (Table 3). All 3 tumors were malignant astrocytomas.

DISCUSSION

Dinitrosopiperazine, as has been previously reported (1), was a potent carcinogen in rats, although apparently less active in Sprague-Dawley rats (as measured by survival of the animals) than in Wistar rats (3). Also, more liver tumors

Table 1

Preparation and properties of dinitrosopiperazines

Nitroso derivatives of piperazines and homologs were prepared by reaction with sodium nitrite in dilute acetic acid. The solids were filtered off and crystallized from hot water.

Compound	Yield from amine (% theoretical)	Melting point	UV absorptivity		
			λ_{nm}	a_M	Solvent
Dinitrosopiperazine	86	155-156°	341	178	H_2O
2,5-Dimethyldinitrosopiperazine	57	169-170	362	163	H_2O
Dinitrosohomopiperazine	55	91-92	347	172	Ethanol
2-Methyldinitrosopiperazine	60	67-68	346	181	H_2O
2,6-Dimethyldinitrosopiperazine	50	87-88	348	173	H_2O

Table 2

Mortality and tumor incidence in rats fed nitrosopiperazines in drinking water

Animals were fed compounds in drinking water 5 days/week. Tumors were determined at postmortem examination.

Chemical	Duration of treatment (wk)	Total dose (mmoles)	No. of animals	Survivors at Wk									No. of TBA[a]
				10	20	30	40	50	60	70	80	90	
Dinitrosopiperazine, 100 mg/liter	50	3.5	15 (M)	15	15	15	15	12	6	3	1	0	15
			14 (F)	14	14	14	14	10	3	2	2	0	13
2,5-Dimethyldinitrosopiperazine, 120 mg/liter	50	3.5	15 (M)	15	15	15	15	9	3	0			15
			15 (F)	15	15	15	10	1	0				15
Dinitrosohomopiperazine, 110 mg/liter	31	2.15	15 (M)	15	15	13	2	0					15
			15 (F)	15	15	14	3	1	0				15
2-Methyldinitrosopiperazine, 110 mg/liter	33	2.3	15 (M)	15	15	12	1	0					15
			15 (F)	15	15	12	1	0					15
2,6-Dimethyldinitrosopiperazine, 120 mg/liter	33	2.3	15 (M)	15	15	10	1	0					15
			15 (F)	15	15	0							15

[a] TBA, tumor-bearing animals.

Table 3

Tumors in Sprague-Dawley rats treated with dinitrosopiperazine, 2,5-dimethyldinitrosopiperazine, dinitrosohomopiperazine, 2-methyldinitrosopiperazine, and 2,6-dimethyldinitrosopiperazine

Animals were fed compounds in drinking water 5 days/week. Duration of treatment same as Table 2. Tumors were determined by gross and microscopic examination.

Compound	Sex	No. of animals necropsied	No. of tumor-bearing animals with tumors of			
			Upper digestive tract	Nasal turbinates	Liver	Brain
Dinitrosopiperazine	M	15	3	14	4	1
	F	14	2	12	6	0
2,5-Dimethyldinitrosopiperazine	M	15	9	15	0	1
	F	15	14	14	0	0
Dinitrosohomopiperazine	M	15	15	15	0	1
	F	15	15	14	0	0
2-Methyldinitrosopiperazine	M	15	14	12	0	0
	F	15	14	13	0	0
2,6-Dimethyldinitrosopiperazine	M	15	13	14	0	0
	F	15	15	14	0	0

were seen in the present experiments than in the Wistar rats, perhaps because the Sprague-Dawley rats survived longer. No liver tumors were seen in the groups treated with the *C*-methyl derivatives of dinitrosopiperazine or with dinitrosohomopiperazine; rats in these groups all died earlier than the dinitrosopiperazine-treated animals.

In all 5 groups that received dinitrosopiperazine or its derivatives, the incidence of tumor-bearing animals was almost 100%, showing that all of these compounds were potent carcinogens, particularly for the olfactory epithelium and esophagus of these rats. We have no basis for speculating on the reason for the susceptibility of these particular tissues to the carcinogenic action of the compounds. Perhaps studies of the distribution and chemical interactions of the compounds in various organs and tissues will give some clues.

These experiments are important chiefly because methyl substitution for hydrogen on certain carbon atoms of dinitrosopiperazine appeared to increase the carcinogenic potency. The presence of an additional methylene in dinitrosohomopiperazine, causing the molecule to be unsymmetrical (although this might not be of importance in itself), made the compound much more carcinogenic than was dinitrosopiperazine. The total dose of dinitrosohomopiperazine was considerably smaller, and yet almost all of the animals had died with tumors at approximately 40 weeks after the beginning of the treatment, at a time when all of the dinitrosopiperazine-treated animals were still alive. By this same measure of dose administered combined with survival pattern, 2,5-dimethyldinitrosopiperazine appeared to be more potent, but probably not significantly more so, than was dinitrosopiperazine. On the

other hand, 2-methyldinitrosopiperazine was considerably more potent than was the parent compound and about as effective as its isomer dinitrosohomopiperazine. The remaining *C*-methyl derivative, 2,6-dimethyldinitrosopiperazine, was even more potent than was the 2-methyl derivative, all of the females being dead with tumors at the 30th week and all but 1 of the males by the 40th week (the females received the same total dose as the males but, being smaller, their dose per unit body weight was more than double that of the males).

Our interpretation of these results is that the rate-limiting step in carcinogenesis by this type of nitrosamine involves loss of one or more hydrogen atoms on carbons adjacent to the *N*-nitroso function. There has been indication of this from the isotope effect on carcinogenicity of nitrosamines, in which the α hydrogen atoms have been replaced by deuterium; the carcinogenic activity in the deuterium-labeled analogs of dimethylnitrosamine (5) and of nitrosomorpholine (W. Lijinsky, H. W. Taylor, and L. Keefer, unpublished results) is greatly reduced by comparison with the parent compound. Therefore, the effect of the additional methylene group in dinitrosohomopiperazine or of the methyl groups in 2-methyldinitrosopiperazine and 2,6-dimethyldinitrosopiperazine is to increase the activity of these α carbon atoms, making the molecule more reactive toward some receptor in the susceptible cells. The lack of effect on carcinogenic activity of the methyl groups in 2,5-dimethyldinitrosopiperazine could be the result of the enhancing effect of one of the methyl groups and the inhibitory effect of the other methyl group α to the nitroso function; this inhibitory effect of α methyl groups has been shown by the lack of carcinogenicity of 2,6-dimethylnitrosopiperidine compared with the potent carcinogen nitrosopiperidine. It is unlikely that the carcinogenicity of 2,5-dimethyldinitrosopiperazine was influenced by the fact that it was a mixture of approximately 25% *cis* and 75% *trans* conformers, since the *syn* and *anti* forms of 2-methylnitrosopiperidine have been shown by Wiessler and Schmähl (8) to have indistinguishable carcinogenic activities.

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