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Comparative tumorigenicity of *N*-nitroso-2-hydroxymorpholine, *N*-nitrosodiethanolamine and *N*-nitrosomorpholine in A/J mice and F344 rats

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N-Nitroso-2-hydroxymorpholine (NHMOR), a genotoxic metabolite of the environmental carcinogens N-nitrosomorpholine (NMOR) and N-nitrosodiethanolamine (NDELA), was assayed for tumorigenicity in A/J mice and F344 rats. Groups of female mice were given NHMOR, NMOR or NDELA in the drinking water over a 10-week period; total doses were 53–55 μmol/mouse. The experiment was terminated after 30 weeks. Whereas NMOR was a potent tumorigen, inducing 20.3 lung tumors/mouse, NHMOR and NDELA were only weakly tumorigenic, giving 1.2 and 1.4 lung tumors/mouse respectively. Groups of female F344 rats were also given these three nitrosamines in drinking water for 50 weeks, as follows: NHMOR, total dose 0.6 mmol/rat; NHMOR, 1.2 mmol; NMOR, 1.1 mmol and NDELA, 5.6 mmol. The experiment was terminated after 120 weeks. NMOR was a potent carcinogen, inducing liver tumors in 100% of the rats. NDELA gave hepatocellular tumors in 70% of the rats. NHMOR was inactive even at the higher dose. The results of this study do not support the hypothesis that NHMOR is a proximate carcinogen of NDELA or NMOR.

Introduction

N-Nitrosodiethanolamine (NDELA*) is one of the most prevalent of the environmental *N*-nitroso compounds. It has been detected in exceptionally high concentrations, up to 3%, in industrial metal grinding fluids, and in the urine of metal grinders (1,2). It has been reported as a common contaminant of cosmetics and toiletries, and is found in tobacco and tobacco smoke (3,4). *N*-nitrosomorpholine (NMOR) has been frequently detected, sometimes at high levels, in the air of rubber and tyre-manufacturing factories (5). It also has been found in cosmetics, toiletries and the fine-cut chewing tobacco used for snuff-dipping (3,6).

The hepatocarcinogenicities of NDELA and NMOR in the rat have been repeatedly confirmed, most recently in an extensive series of dose–response studies which demonstrated that NMOR is more potent than NDELA (7–10). NMOR is one of the most carcinogenic of the *N*-nitroso compounds which occur in the environment.

N-Nitroso-2-hydroxymorpholine (NHMOR) has been detected as a metabolite of NDELA in rat liver 9000 *g* supernatant (11,12). *In vivo*, it is apparently oxidized to *N*-nitroso-2-hydroxyethylglycine (NHEG), which is a urinary metabolite of NDELA (see Figure 1) (13,14). Up to 95% of NDELA administered *per os* to rats is excreted unchanged (14,15). The detection of NHMOR as a metabolite of NDELA, and its mutagenicity in *Salmonella typhimurium*, with or without activation, suggested that it might be a proximate carcinogen of NDELA (12,16). Alcohol dehydrogenase activation of NDELA produced mutations in *S.typhimurium*, which is consistent with this hypothesis (17). NHMOR also caused single-strand breaks in primary rat hepatocytes and in rat liver *in vivo* (16). It reacted with deoxyguanosine to produce a cyclic 1,N^2-glyoxal adduct (18) and transferred its nitroso group to secondary amines (11,19).

In contrast to NDELA, NMOR is extensively metabolized in the rat, with only 1–2% of the dose being excreted unchanged in urine (20). Established pathways of metabolism include α-hydroxylation, β-hydroxylation to NHMOR and NDELA, and denitrosation (20–23). Several lines of evidence favor α-hydroxylation of nitrosamines as an important metabolic activation mechanism (20,24), but the direct genotoxicity of NHMOR suggested that β-hydroxylation of NMOR may also be involved in activation of NMOR.

In the present study, we have evaluated the comparative tumorigenic activities of NDELA, NMOR and NHMOR in A/J mice and F344 rats in order to assess the possible role of NHMOR in carcinogenesis of NDELA and NMOR.

Materials and methods

Chemicals

NHMOR was synthesized and purified as previously described (12). It was pure according to TLC and NMR analysis. NDELA and NMOR were also prepared and purified as described (10,25,26).

Animals

Female A/J mice, age 8–10 weeks were obtained from Jackson Laboratories, Bar Harbor, ME. They were housed in groups of 10 in solid-bottom polycarbonate cages with hardwood bedding. The animals were kept at 20 ± 2°C and 50 ± 10% (SD) relative humidity with a 12 h light:dark cycle. They were given NIH-07 diet *ad libitum*.

Female F344 rats, age 8 weeks, were born and raised in the Frederick Cancer Research Facility. They were maintained in cages of four within a barrier, and fed Purina Rodent Chow pellets *ad libitum*.

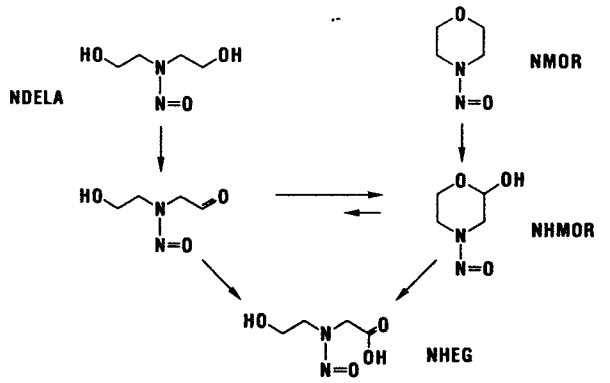

Fig. 1. Metabolism of NDELA and NMOR by oxidation at carbons β- to the *N*-nitrosamino group. NHEG and NHMOR have been identified as metabolites.

*Abbreviations: NDELA, *N*-nitrosodiethanolamine; NMOR, *N*-nitrosomorpholine; NHMOR, *N*-nitroso-2-hydroxymorpholine; NHEG, *N*-nitroso-2-hydroxyethyl glycine.

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Bioassay in A/J mice

The mice were assigned to groups as summarized in Table I. For the first 10 weeks of the bioassay, 0.2 μmol/ml nitrosamines were added to the drinking water. Fresh solutions of the nitrosamines in amber bottles were provided twice weekly. Water consumption was measured twice weekly during the 10 weeks of nitrosamine treatment. After these 10 weeks were complete, animals received tap water for the next 20 weeks. They were then killed by CO_2 immersion, and lung adenomas were counted. Statistical significance was assessed by the χ^2 test and Student's *t*-test.

Bioassay in F344 rats

Rats were assigned to groups as indicated in Table II. Each cage of four rats was given 80 ml of the appropriate nitrosamine solution, or tap water alone for the control group, on each of 5 days per week. All of this was comsumed with little spillage. On the other 2 days, water was given *ad libitum* to allow the rats to make up any water deficiency that had been incurred. The treatments continued for 50 weeks, after which the animals were maintained until they died or were killed when moribund. Survivors were killed at weeks 120–124. The animals were necropsied and all lesions, together with major organs and tissues, were fixed in formalin, embedded in paraffin, sectioned and stained for histologic examination.

Table I. Lung tumors in A/J mice treated with NDELA, NMOR or NHMOR in drinking water[a]

Group	No. of mice	Dose (μmol/ml)	H_2O consumption (ml/day)	Total dose (μmol)	% Mice with lung tumors	Lung tumors mouse (mean ± SD)
Control	40	–	3.9 ± 0.4	–	40	0.5 ± 0.6
NDELA	40	0.2	4.0 ± 0.4	55	70[b,c]	1.4 ± 1.2[b,c]
NMOR	40	0.2	3.9 ± 0.4	54	100[b]	20.3 ± 6.0[b]
NHMOR	40	0.2	3.8 ± 0.4	53	60[c]	1.2 ± 1.3[b,c]

[a]Nitrosamines were administered in the drinking water for 10 weeks. Twenty weeks later, animals were killed and lung tumors counted.
[b]Significantly more tumorigenic than control, $P < 0.01$.
[c]Significantly less tumorigenic than NMOR, $P < 0.01$.

Table II. Survival of female F344 rats treated with NDELA, NMOR and NHMOR in drinking water

Treatment (dose, mg/l)	Total dose (mmol)	Survivors at week: 0	10	20	30	40	50	60	70	80	90	100	110	120
Control		20	20	20	20	20	20	20	19	18	17	17	9	5
NDELA (150)	5.6	20	20	20	20	20	20	19	19	18	16	11	6	0
NMOR (26.5)	1.1	20	20	20	20	20	20	15	5	0				
NHMOR (15)	0.6	20	20	20	20	20	20	20	20	20	19	17	11	6
NHMOR (30)	1.2	20	20	20	20	20	20	20	20	19	18	15	9	4

Results

Table I summarizes the results of the assay in A/J mice. NMOR was clearly the most tumorigenic compound, incuding 20.3 lung tumors/mouse. NDELA and NHMOR showed comparably weak but statistically significant tumorigenic activities compared with controls.

Tables II and III summarize the results of the bioassay in F344 rats. As shown in Table II, NMOR at 26.5 mg/l of water, corresponding to a weekly dose of ~23 μmol/rat, was by far the most carcinogenic treatment, leading to death of half of the animals by week 64 and to death of all by week 73. All of these rats had neoplasms in the liver, hepatocellular carcinomas or hemangiosarcomas, often both, more than half of which metastasized to the lungs. Incidences of other neoplasms were small (Table III) and probably not related to the treatment. The NDELA treatment was less effective, although the molar dose delivered to the animals was five times that of NMOR. Half of the NDELA-treated rats were dead at week 101 and the last two survivors were killed at week 120. More than half of them had liver neoplasms, all but one hepatocellular and few of these had metastasized. There were few other neoplasms that were considered potentially treatment related in the group of rats given NDELA, but one was a tubular cell adenoma of the kidney.

At week 100, most of the rats given NHMOR and most of the control rats were alive; ≥20% in these groups survived until they were killed. No treatment-related neoplasms were seen in the groups given NHMOR at either dose level. Tumors were present in most of these animals, but they are considered spontaneous in F344 rats and did not differ significantly in incidence from the controls. They included neoplasms of the pituitary, adrenal medulla, thyroid C-cells, mammary gland, pancreas islets and mononuclear cell leukemia.

Discussion

The results of this study do not support the hypothesis that NHMOR is involved in the tumorigenic metabolic activation of

Table III. Tumors in female F344 rats treated with NDELA, NMOR or NHMOR in drinking water

Treatment (dose, mg/l)	Number of animals with tumors: Liver: Hepatocellular	Liver: Hemangiosarcoma	Lung	Thyroid follicular cell	Other[a]
Control	0	0	0	0	
NDELA (150)	14	1	1	0	Kidney adenoma 1 Brain astrocytoma 1 Adrenal cortex 1
NMOR (26.5)	19	10	1	2	Kidney adenoma 1 Adrenal cortex 1 Brain astrocytoma 1
NHMOR (15)	1	0	0	0	Thymus lymphoma 1 Myxosarcoma 1 Adrenal cortex 1
NHMOR (30)	0	0	1	0	Thymus lymphoma 2 Bone osteosarcoma 1 Mammary gland carcinoma 2

[a]These are tumors not usually seen in untreated F344 rats.

NDELA or NMOR. In mice and rats, NHMOR was either very weakly active or inactive at the doses tested, whereas NMOR was strongly tumorigenic in both species. These results indicate that NHMOR is a detoxified metabolite of NMOR. In rats, 40–60% of NMOR metabolism proceeds by this pathway, as determined by measuring levels of NHEG in urine (27). β-Oxidation of NDELA to NHMOR *in vivo* in the rat accounts for <10% of the dose, based on excretion of NHEG in urine (13,14,27). In the bioassay in rats, the higher dose of NHMOR was 20% of the dose of NDELA. Thus, if NHMOR were a proximate carcinogen of NDELA, one would have expected to see at least a comparable tumor yield from the two compounds. This was not observed, and there was no indication that NHMOR was carcinogenic in the rat. In mice, equivalent doses of NHMOR and NDELA gave similar weak responses, which also indicates that NHMOR is not a proximate tumorigen of NDELA in A/J mice.

Previous studies have shown that NHMOR is an effective inducer of single-strand breaks in rat liver, and is mutagenic toward *S.typhimurium* with and without rat liver 9000 *g* supernatant activation (12,16). The mechanisms of single-strand breakage and mutagenicity by NHMOR are not known, but may be unrelated to carcinogenesis by this compound since no liver tumors were observed in the treated rats.

Sterzel and Eisenbrand have suggested that sulfation of NDELA may be an activation mechanism, since induction of single-strand breaks was abolished by a sulfotransferase inhibitor (28). NDELA sulfate could cause 2-hydroxyethylation of DNA according to the mechanism proposed by Michejda and co-workers (29). Support for this mechanism has been obtained by the tentative identification of 2-hydroxyethyl adducts in liver DNA of NDELA-treated rats (14). Further studies are required to determine whether this, or other activation mechanisms, are involved in the expression of NDELA carcinogenicity.

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