Jpn. J. Cancer Res. (Gann), 79, 698–704 ; June, 1988

Carcinogenic Potency of N-Nitrosomethyl(2-hydroxypropyl)amine and Other Metabolic Relatives of N-Nitrosobis(2-hydroxypropyl)amine by Single Intraperitoneal Injection on the Lung of Rats

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The carcinogenic effects of a single intraperitoneal injection of N-nitrosobis(2-hydroxypropyl)-amine (BHP) or its metabolic relatives, N-nitrosomethyl(2-hydroxypropyl)amine (MHP), N-nitrosobis(2-oxopropyl)amine (BOP), N-nitroso(2-hydroxypropyl)(2-oxopropyl)amine (HPOP) and N-nitroso-2,6-dimethylmorpholine (NDMM), were studied in male Wistar rats. The main target organ of these nitrosamines proved to be the lung, followed by the thyroid. Lung lesions were induced in a dose-dependent manner with total lung tumor incidences reaching 55% to 100%. BHP, MHP, HPOP and NDMM all caused lung carcinomas to develop (22% to 44% incidence), whereas BOP was only associated with adenomas. On the basis of dose administered and incidence of carcinomas, MHP appeared to be the most potent lung carcinogen of the five nitrosamines investigated. Smaller numbers of neoplasms were also induced in the kidney, urinary bladder, esophagus and intestine at differing rates by these nitrosamines.

Key words: N-Nitrosopropanolamines — Lung carcinogenesis — Rat

It has been reported that lung neoplasia can be induced by both oral or a single intraperitoneal administration of BHP to rats.[1, 2] Histologically, the induced peripheral lung carcinomas are similar to those which are reportedly increasing among nonsmoking human populations,[3, 4] and since BHP is an environmental contaminant of commercial samples of diisopropanolamine and triisopropanolamine, both of which are used in manufacturing processes and cosmetic preparations,[5] this carcinogen may be of direct significance to cancer in man. Therefore the biological effects of BHP and metabolically related compounds after systemic administration to experimental animals have attracted attention. In the present study, we investigated the carcinogenic response of Wistar rats to a single intraperitoneal injection of BHP, MHP, BOP, HPOP and NDMM, with particular emphasis being placed on resultant lung lesion development.

Abbreviations: BHP, N-nitrosobis(2-hydroxypropyl)amine; MHP, N-nitrosomethyl(2-hydroxypropyl)amine; BOP, N-nitrosobis(2-oxopropyl)-amine; HPOP, N-nitroso(2-hydroxypropyl)(2-oxopropyl)amine; NDMM, N-nitroso-2,6-dimethylmorpholine.

MATERIALS AND METHODS

A total of 127 male, 7-week-old Wistar rats (Shizuoka Laboratory Animal Center, Shizuoka) were used. The number of rats used in each group is shown in Table I. All the N-nitrosamines examined were prepared as described previously[6]; the purity in each case was more than 99%, as determined by high-pressure liquid chromatography. The nitrosamines were stored at −20° prior to use.

Rats were housed 6 to a wire cage in an air-conditioned room at 24° and maintained on a commercial stock diet, Oriental MF (Oriental Yeast Ind., Tokyo) *ad libitum*. Rats were randomly divided into twelve groups as shown in Table I. Each compound was dissolved in saline and administered as a single intraperitoneal injection at the dose shown in Table I. The ratios of the doses of nitrosamines to the 50% lethal dose which was determined by Weil's method[7] are shown in Table I. All surviving animals were sacrificed under ether anesthesia 55 weeks after the administration of the nitrosamines and completely autopsied.

Organs were fixed in 10% buffered formalin, and processed for histological examination by conventional methods.

The numbers and diameters of lung lesions arising in bronchiolo-alveolar tissue, including hyperplasia, adenoma and carcinoma, were analyzed with an Imagelyzer model HTB-C995 (Hamamatsu Television Co., Ltd., Shizuoka) connected with a Desktop Computer System-45 (Hewlett-Packard, Colorado).

The *t*-test was used for statistical comparison of body weights, organ weights, and numbers and areas of lung lesions between groups. Tumor incidence was compared by the χ^2 test.

Results

Changes in Body and Organ Weights Initial and final body weights and final organ weights are shown in Table I. The final body weights of animals in groups 2 to 12 were significantly lower than those of group 1; in particular those of rats in groups 8 and 9 showed striking decreases compared to group 1, suggesting a toxicity of HPOP at the doses used. Liver weights but not liver-to-body weight ratios of rats in groups, 3, 4, 6, 7, 8, 9, 10, 11 and 12 were also significantly decreased as compared to group 1. Lung weights and lung-to-body weight ratios increased in all groups receiving nitrosamines due to the tumors that developed. Kidney weights of rats in group 5 were significantly increased (due to kidney tumors) compared to group 1.

Lung Tumor Incidences The incidences of lung tumors induced by the different nitrosamines are summarized in Table II. The lesions were diagnosed histologically on the basis of their characteristic features as described previously.[8-11] All nitrosamines tested here induced lung tumors in a dose-dependent manner and the total tumor incidence

Table I. Treatment Groups, Animal Numbers, Body and Main Organ Weights

Group No.	Treatment	Dose (mmol/kg BW)	Dose Ratio to LD_{50}	No. of rats Initial	No. of rats Final	Body weight (mean±SD)(g) Initial	Body weight (mean±SD)(g) Final	Organ weight (mean±SD)(g) (% of BW) Lung	Liver	Kidney
1	Saline	——	——	10	10	185±5.8	444±20	2.4±0.2 (0.5)	11.4±0.6 (2.6)	1.3±0.1 (0.3)
2	BHP	6.17	0.18	10	10	192±15.0	394±42[b]	3.8±1.0[c] (1.0)	10.8±1.4 (2.7)	1.3±0.1 (0.3)
3	BHP	18.52	0.55	10	10	193±9.9	374±37[c]	3.8±0.5[c] (1.0)	10.3±1.4[d] (2.6)	1.3±0.2 (0.3)
4	MHP	0.38	0.25	10	9	185±8.4	363±44[c]	3.9±0.8[c] (1.1)	9.3±1.0[c] (2.6)	1.4±0.2 (0.4)
5	MHP	0.75	0.50	11	9	187±9.6	373±47[c]	4.8±2.6[c] (1.3)	9.8±2.4 (2.6)	2.6±1.3[b,d] (0.7)
6	BOP	0.34	0.11	10	10	192±7.9	385±22[c]	3.2±0.9[c] (0.8)	9.7±0.9[c] (2.5)	1.2±0.1[c] (0.3)
7	BOP	0.67	0.22	11	10	189±5.3	381±26[c]	4.0±1.1[c] (1.0)	9.7±0.8[c] (2.5)	1.3±0.3 (0.3)
8	HPOP	1.33	0.19	11	10	190±4.2	227±29[c]	5.5±3.1[c] (2.4)	5.6±0.9[c] (2.5)	4.9±12.4 (2.2)
9	HPOP	2.65	0.38	10	6	192±5.2	230±31[c]	3.8±1.1[b] (1.7)	6.2±1.4[c] (2.7)	1.2±0.2 (0.5)
10	NDMM	0.26	0.13	12	12	189±6.2	385±20[c]	2.5±0.3 (0.6)	9.8±0.4[c] (2.5)	1.2±0.4 (0.3)
11	NDMM	0.52	0.26	11	11	194±4.7	378±43[c]	3.1±0.4[e,c] (0.8)	9.8±1.3[b] (2.6)	1.2±0.1[c] (0.3)
12	NDMM	1.04	0.52	11	9	191±12.0	378±23[c]	3.1±0.4[e,f] (0.8)	10.4±1.0[c] (2.7)	1.4±0.3 (0.3)

a) $P<0.05$ compared with group 1.
b) $P<0.01$ compared with group 1.
c) $P<0.001$ compared with group 1.
d) $P<0.05$ compared with group 4.
e) $P<0.001$ compared with group 10.
f) $P<0.01$ compared with group 10.

Table II. Incidence of Lung Tumors in Rats Treated with BHP, MHP, BOP, HPOP or NDMM

Group No.	Treatment	Dose (mmol /kg BW)	Effective no. of rats[a]	Tumor incidence (%)						
				Total	Bronchiolo-alveolar region[b]					Bronchial papilloma
					Total	A	AC	ASC	S	
1	Saline	——	10	0 (0)	0 (0)	0	0	0	0	0
2	BHP	6.17	10	9 (90)	6 (60)	4	3	1	0	6
3	BHP	18.52	10	10(100)	9 (90)	7	8	4	0	7
4	MHP	0.38	9	4 (44)	2 (22)	0	1	0	1	2
5	MHP	0.75	9	8 (89)	6 (67)	4	3	1	0	2
6	BOP	0.34	10	4 (40)	1 (10)	1	0	0	0	3
7	BOP	0.67	10	10(100)[c]	10(100)[c]	10[c]	0	0	0	5
8	HPOP	1.33	10	7 (70)	6 (60)	5	2	1	1	1
9	HPOP	2.65	6	6(100)	6(100)	3	3	0	0	1
10	NDMM	0.26	12	2 (17)	0 (0)	0	0	0	0	2
11	NDMM	0.52	11	2 (18)	1 (9)	1	0	0	0	1
12	NDMM	1.04	9	5 (55)	4 (44)	2	1	1	0	1

a) Based on histological examination.
b) A, adenoma; AC, adenocarcinoma; ASC, adenosquamous carcinoma; S, squamous cell carcinoma.
c) $P<0.05$ compared with group 6.

Table III. Numbers and Areas of Lung Tumors in Rats Treated with BHP, MHP, BOP, HPOP or NDMM[a]

Group No.	Treatment	Dose (mmol /kg BW)	Hyperplasia			Adenoma			Carcinoma[b]		
			Incidence (%)	No. /rat	Area ($\times 10^{-2}$ cm^2)	Incidence (%)	No. /rat	Area ($\times 10^{-2}$ cm^2)	Incidence (%)	No. /rat	Area ($\times 10^{-2}$ cm^2)
1	Saline	0	0	0	0	0	0	0	0	0	0
2	BHP	6.17	100	6.2 ± 5.1[c]	1.3 ± 0.5[d]	40	1.1 ± 1.4	1.5 ± 0.6	40	0.8 ± 1.1	10.3 ± 11.9
5	MHP	0.75	67	2.8 ± 3.1	2.3 ± 1.9	44	0.6 ± 0.7	2.3 ± 1.9	44	0.6 ± 0.7	52.8 ± 96.2
7	BOP	0.67	100	5.3 ± 3.1[c]	2.9 ± 1.4	100	1.7 ± 0.9[d]	5.3 ± 6.3	0	0	0
8	HPOP	1.33	90	1.7 ± 1.3	1.7 ± 0.7	50	0.7 ± 0.8	3.2 ± 2.8	40	0.5 ± 0.7	8.5 ± 12.4
12	NDMM	1.04	89	4.0 ± 2.3[c]	1.0 ± 1.3[d]	22	1.0 ± 0	1.0 ± 1.4	22	1.0 ± 0	17.0 ± 22.6

a) Values represent mean $\pm$ standard deviation.
b) Including adenocarcinoma, adenosquamous cell carcinoma and squamous cell carcinoma.
c) $P<0.05$ compared with HPOP group.
d) $P<0.05$ compared with MHP group.

reached 55% to 100% in the higher or highest dose group for each carcinogen. Comparing the total tumor incidences among groups 3, 5, 7, 9 and 12, group 7 showed an equal or a higher yield than any other group despite the fact that the absolute dose given was the lowest of all. Group 5, given the second lowest dose, showed an approximately equal incidence to that in group 2. Histologically, the spectrum of induced lung tumors was uniform among all nitrosamine-treated groups with the exception of that given BOP, in which no carcinomas were observed. Bronchiolo-alveolar adenoma was the commonest type of tumor in all nitrosamine-treated groups with incidences being dose-dependent in the groups given BHP, MHP, BOP and NDMM. That in group 7 was the highest at 100%. The incidences of adenocarcinomas, the predominant type of malignant lesion induced, also showed dose-dependence in the groups given BHP, MHP and HPOP. Adenosquamous carcinomas were induced in a dose-related way by BHP, but they were rare or absent in the other nitrosamine-treated groups. Squamous cell carcinomas were occasionally observed in groups 4 and 8. Bronchial papillomas, the other type of tumor common to all nitro-

Table IV. Tumor Distribution in Other Organs of Rats Treated with BHP, MHP, BOP, HPOP or NDMM

Group No.	Treatment	Dose (mmol /kg BW)	Effective no. of rats	No. of rats with tumors (%)	Tumor incidence (%)						
					Thyroid			Kidney			Other sites
					Total	A[a]	C[b]	Total	A[c]	C[d]	
1	Saline	——	10	0 (0)	0 (0)	0	0	0 (0)	0	0	
2	BHP	6.17	10	8 (80)	7 (70)	7	0	1(10)	1	0	
3	BHP	18.52	10	10(100)	10(100)	10	3	2(20)	0	2	1[e]
4	MHP	0.38	9	3 (33)	3 (33)	3	0	0 (0)	0	0	
5	MHP	0.75	9	6 (67)	6 (67)	5	2	2(22)	0	2	1[f]
6	BOP	0.34	10	3 (30)	3 (30)	3	0	1(10)	0	1	
7	BOP	0.67	10	7 (70)	6 (60)	5	3	1(10)	0	1	1[g]
8	HPOP	1.33	10	9 (90)	9 (90)	9	4	2(20)	0	2	2[h,i]
9	HPOP	2.65	6	6(100)	5 (83)	3	2	1(17)	0	1	1[h]
10	NDMM	0.26	12	1 (8)	1 (8)	1	0	0 (0)	0	0	
11	NDMM	0.52	11	1 (9)	1 (9)	1	0	0 (0)	0	0	
12	NDMM	1.04	9	3 (33)	1 (11)	0	1	2(22)	0	2	

a) Papillary or follicular adenoma; *b*) papillary or follicular adenocarcinoma;
c) adenoma; *d*) pelvic transitional cell carcinoma; *e*) esophageal squamous cell carcinoma;
f) urinary bladder transitional cell carcinoma; *g*) esophageal papilloma;
h) urinary bladder papilloma; *i*) intestinal carcinoma.

samine-treated groups, were observed predominantly in animals given BHP or BOP, the incidences increasing with increasing dose.

Number and Area of Lung Lesions To further compare the carcinogenic activities of the nitrosamines tested, groups 2, 5, 7, 8 and 12, having approximately equal values for incidences of carcinomas (with the exception of BOP), were chosen and the numbers and areas of preneoplastic and neoplastic lesions appearing in bronchiolo-alveolar lung regions were calculated (see Table III). The incidence of hyperplasias reached 100% in groups 2 and 7, whereas that in group 5 was 67%. Numbers of hyperplasias per rat in groups 2, 7 and 12 were significantly higher than that in group 8. Numbers and areas of both hyperplasias and adenomas were highest in group 7. Size of carcinomas, as reflected in area occupied, was higher in group 5 than in groups 2 and 8. MHP (group 5) thus demonstrated carcinogenic activity in the bronchiolo-alveolar region similar to or stronger than those of BHP (group 2), HPOP (group 8) and NDMM (group 12), even at a much lower dose.

Tumor Incidence in Other Organs The incidences and distribution of tumors induced in sites other than the lung are summarized in Table IV. Tumors developed in the thyroid, kidney, urinary bladder, esophagus and intestine, the thyroid being the second most frequent site of tumor development. Thus, all the nitrosamines induced adenomas and carcinomas of the thyroid, and a dose-dependent relationship was observed in the groups given BHP, MHP and BOP. The incidence of kidney tumors was low, with no dose-dependence being evident. Tumor in other sites were observed sporadically in some nitrosamine-treated groups.

Discussion

In the present study, significant carcinogenic effects were demonstrated for single intraperitoneal injections of BHP, MHP, BOP, HPOP and NDMM in male Wistar rats. The commonest site of tumor development for all these nitrosamines was the lung, followed by the thyroid, and clear dose-dependent induction of lesions was observed in both of these organs. Compared to previous reports,[12–15] the tumor distribution in the present study differed considerably: earlier studies of propylnitrosamine carcinogenesis revealed that the nasal cavity, esophagus and liver were the most commonly affected organs. However, the carcinogens had been administered to

other rat strains, either orally or subcutaneously, at much higher doses than those used in the present study. That this may be of importance is suggested by the report that oral administration of large doses of BHP induced liver and esophagus tumors in addition to lung lesions,[16] whereas small doses of the same carcinogen given as a single intraperitoneal injection result in selective induction of lung neoplasia.[1] These earlier observations in conjunction with the present findings thus indicate that a small ip dose of BHP or the other four related nitrosamines tested might be metabolized to carcinogenic forms particularly potent for the lung. Differences in quantitative and/or qualitative metabolic degradation with the route of administration, variations in strain-related enzymatic activation or other intrinsic or extrinsic factors might all be involved[17, 18] in determining target organ specificity. Nevertheless, it seems clear that BHP and the related compounds MHP, BOP, HPOP and NDMM induce lung tumors by essentially similar mechanisms in each case.

Studies of the metabolism of the nitrosamines used in the present study demonstrated that they can be interconverted in both hamsters and rats, although only a small percentage of any particular nitrosamine administered normally appears as the others in the various body compartments.[6, 19-21] Thus in Wistar rats, BHP, HPOP, glucuronides of BHP and HPOP, MHP, BOP and unknown metabolites are all detected in the blood, liver, lung, kidney and urine after a single intraperitoneal injection of BHP.[6] In the lung, 3.1% and 0.15% of BHP administered are reportedly converted to HPOP and MHP, respectively, but no BOP was detected.[6]

Mutagenic assay by Ames's test demonstrated that BHP, MHP and NDMM are all activated to mutagens by liver S9 either from untreated or polychlorinated biphenyl(PCB)-treated rats.[22] HPOP and BOP are weakly mutagenic in the absence of S9, but their mutagenicities are highly activated by rat liver S9.[22, 23] Further, only MHP, but not BHP, HPOP, BOP or NDMM, is activated to mutagens by lung S9 from PCB-treated rats.[23] These findings indicate that BHP and related nitrosamines are all metabolized in the liver and MHP can in addition be further metabolized in the lung. Together with these findings, the present result that MHP exerts the strongest carcinogenic activity for the lung on the basis of dose and incidence of bronchiolo-alveolar carcinomas thus suggests that this form acts as a more proximate carcinogen in rat lung carcinogenesis by BHP.

The present result that BOP, which might have a direct mutagenic potential,[23] induced the highest incidences of lung hyperplasias and adenomas even though it induced no adenocarcinomas is intriguing. The possibility that BOP exerts a strong initiating activity but a weak promoting one, while MHP exerts strong initiating and promoting ones would be worth examining.

Recently, we reported that bronchiolo-alveolar hyperplasia might be a preneoplastic lesion for BHP-induced lung adenocarcinomas on the basis of comparative histochemical analysis using γ-glutamyltranspeptidase.[24] Enzyme alterations in this type of lesion have also been reported by other workers using different markers.[25, 26] Bronchiolo-alveolar adenomas and adenocarcinomas are thought to be histogenetically derived from Clara cells or type II pneumocytes.[10, 25, 26] It has been shown that Clara cells possess strong activity of cytochrome P450, whereas type II pneumocytes have only weak activity[27-30] and therefore it would appear likely that the former are primarily responsible for metabolic activation of MHP to the ultimate carcinogenic form. However, since the lung contains over 40 different types of cells[31] whose roles have not been thoroughly established, a great deal of further study will be required before a complete understanding of the carcinogenic mechanisms of BHP and metabolically related compounds in the lung of rats can be established.

ACKNOWLEDGMENTS

This study was supported in part by a Grant-in-Aid for Cancer Research from the Ministry of Health and Welfare of Japan and a Grant-in-Aid from the Ministry of Health and Welfare for the Comprehensive 10-Year Strategy for Cancer Control, Japan.

(Received March 5, 1988/Accepted April 25, 1988)

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