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Suppressive Effects of *S*-Methyl Methanethiosulfonate on Promotion Stage of Diethylnitrosamine-initiated and Phenobarbital-promoted Hepatocarcinogenesis Model

Shigeyuki Sugie,[1,2,4] Kiyohisa Okamoto,[1] Masami Ohnishi,[1] Hiroki Makita,[1] Toshihiko Kawamori,[1] Tomoyuki Watanabe,[1] Takuji Tanaka,[1] Yasushi K. Nakamura,[3] Yoshiyuki Nakamura,[3] Isao Tomita[3] and Hideki Mori[1]

[1]*Department of Pathology,* [2]*Institute of Laboratory Animals, Gifu University School of Medicine, 40 Tsukasa-machi, Gifu 500 and* [3]*School of Pharmaceutical Sciences, University of Shizuoka, 52-1 Yada, Shizuoka 422*

Modifying effects of *S*-methyl methanethiosulfonate (MMTS) on diethylnitrosamine (DEN)-initiated and phenobarbital (PB)-promoted hepatocarcinogenesis were examined in rats. Five-week-old male F344 rats were divided into 8 groups. After a week, groups 1–5 were given DEN (100 mg/kg body weight, i.p.) once a week for 3 weeks, whereas groups 6–8 received vehicle treatment. Group 2 was given 100 ppm MMTS containing diet in the initiation phase. From 4 weeks after the start of experiment, groups 3 and 5 were fed MMTS, and groups 1–3 and 7 received drinking water containing 500 ppm PB. Group 6 was given MMTS diet alone throughout the experiment (24 weeks). The incidences of hepatocellular adenoma and total liver tumors were significantly smaller in group 3 than those of group 1. The average numbers of hepatocellular adenoma, carcinoma and total tumors in group 3 were significantly smaller than in group 1. Glutathione *S*-transferase placental form-positive foci were also significantly decreased by MMTS treatment in the promotion phase. MMTS treatment in the initiation or promotion phase reduced ornithine decarboxylase activity in the liver of rats given DEN. The antioxidant activity against lipid peroxidation of MMTS was confirmed in tests with rabbit erythrocyte membrane ghosts or rat hepatocytes. These results suggest that MMTS is a promising chemopreventive agent for liver neoplasia when concurrently administered with PB.

Key words: *S*-Methyl methanethiosulfonate — Hepatocarcinogenesis — Chemoprevention — Ornithine decarboxylase — Antioxidant

Epidemiologic studies suggest that the consumption of green and yellow vegetables is inversely related to cancer risk.[1-4] Organosulfur compounds that are present abundantly in a group of cruciferous vegetables or allium species have been shown to possess chemopreventive properties.[5] We have reported protective effects of benzyl isothiocyanate and benzyl thiocyanate on diethylnitrosamine (DEN)-induced hepatocarcinogenesis and methylazoxymethanol acetate-induced intestinal carcinogenesis in rats.[6,7] Reddy *et al.* who reported chemopreventive properties of several organosulfur compounds on azoxymethane-induced colon carcinogenesis demonstrated that phase I and phase II enzymes, including glutathione *S*-transferase (GST), were increased in the liver of rats exposed to organosulfur compounds.[8] GST is a phase II enzyme that has been reported to be involved in the metabolism, detoxification and elimination of carcinogens. Recently, Nakamura *et al.* isolated *S*-methyl methanethiosulfonate (MMTS) (Fig. 1) from cauliflower, *Brassica oleracea* L. var. *botrytis*, and found that it inhibits the UV-induced mutation in *Escherichia coli B/r* WP2 by activation of the excision-repair systems.[9] We have examined the modifying effect of MMTS on large bowel carcinogenesis and found that this organosulfur compound has a strong protective effect.

In this study, we examined the possible chemopreventive effects of MMTS on a DEN/phenobarbital (PB)-induced hepatocarcinogenesis model and on the induction of GST placental form (GST-P)-positive foci, which are preneoplastic lesions of the liver. Since, ornithine decarboxylase (ODC) activity has been shown to regulate cell proliferation and to play a role in tumor promotion,[10] the activity of this enzyme was also assayed in liver tissues. Furthermore, the antioxidant activity of MMTS was examined.

MATERIALS AND METHODS

Animals, diet and chemicals Weanling male F344 rats (Shizuoka SLC, Co., Shizuoka) were used. CE-2 (CLEA Japan Inc., Tokyo) was used as a basal diet. DEN and MMTS were purchased from Nacalai Tesque Inc., Kyoto and Sigma Chemical Co., Inc., St. Louis, MO, respectively. PB was obtained from Maruishi Pharm. Co., Osaka.

[4] To whom correspondence should be addressed.

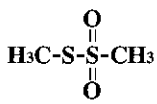

Fig. 1. Molecular structure of *S*-methyl methanethiosulfonate (MMTS)

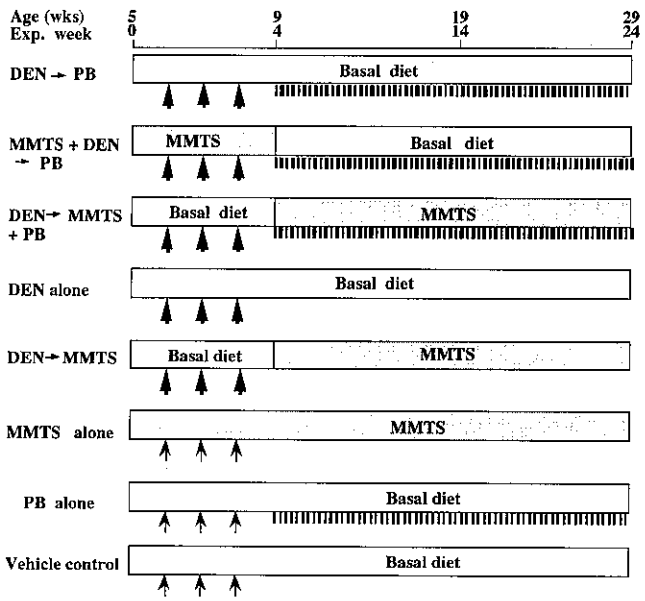

Fig. 2. Experimental design. ↓: DEN 100 mg/kg body weight, once a week for 3 weeks, ↑: Saline, ▥ PB 500 ppm in drinking water, ▨ MMTS 100 ppm in diet.

Determination of dose of MMTS At 5 weeks of age, groups of male F344 rats (6/group) were administrated various levels of MMTS by gavage to determine the appropriate dose for long-term study. MMTS was tested at dose levels of 10,000, 5000, 1000 and 500 ppm, and the LD_{50} value was found to be 1000 ppm. We used 100 ppm MMTS (1/10 of LD_{50}) for testing the effect on hepatocarcinogenesis.

Experimental procedure The experimental design is shown in Fig. 2. A total of 135 rats, 5 weeks of age, were divided into 8 groups: group 1, 20 rats for DEN and 500 ppm PB; group 2, 20 rats for DEN and 100 ppm MMTS in the initiation phase and 500 ppm PB; group 3, 21 rats for DEN and 500 ppm PB and 100 ppm MMTS in the promotion phase; group 4, 24 rats for DEN alone; group 5, 20 rats for DEN and MMTS in the promotion phase; group 6, 8 rats for MMTS alone; group 7, 8 rats for PB alone; and group 8, 14 rats for vehicle control. All animals were housed in wire cages (3 rats/cage) under controlled humidity (50±10%), lighting (12 h light/dark cycle) and temperature (23±2°C). They had free access to water and diets. The experimental diets with MMTS were prepared weekly and stored in a cold room.

Animals in groups 1 through 5 were given i.p.-injections of DEN (100 mg/kg body weight) once a week for three weeks from one week after the start of the experiment, and groups 6 through 8 received i.p.-injections of saline (vehicle). Rats in groups 2 and 6 were given 100 ppm MMTS-containing diet from the start of the experiment and the animals in the other groups were given the basal diet. Animals in groups 1, 4 and 8 were fed the basal diet alone throughout the experiment (24 weeks). Animals in group 2 were transferred from the experimental diet to the basal diet and continued on this regimen to the end of experiment. Groups 1, 2, 3 and 7 received drinking water containing 500 ppm PB from one week after the end of carcinogen or vehicle treatment. Groups 3 and 5 were fed the diet with 100 ppm MMTS from one week after the end of DEN or vehicle treatment. At the termination of the experiment, complete autopsies were performed after the animals had been killed by decapitation under ether anesthesia. At autopsy, the location, number and size of liver tumors were recorded. Liver tissues were sliced into three pieces from each lobe. One set of slices was fixed in cold acetone and another set was fixed in 10% buffered formalin, embedded in paraffin blocks, and processed for routine histological observation with the use of hematoxylin and eosin stain. The sections from acetone-fixed liver were stained for GST-P stain. Immunohistochemical staining for GST-P was carried out using the avidin-biotin-peroxidase complex method (Vectastain ABC kit, Vector Lab. Inc., Burlingame, CA). Anti-GST-P antibody was kindly provided by Dr. K. Satoh, Hirosaki University School of Medicine, Hirosaki. The areas of GST-P-positive foci and number of foci/cm^2 were measured by means of an image analyzer with a microscope (IPAP, Sumitomo Chemical Co., Ltd., Osaka). GST-P-positive lesions composed of more than 11 cells were recognized as altered liver cell foci.

Measurement of ODC activity At necropsy, macroscopic non-tumor tissues from the liver of DEN-treated animals, and liver tissues of vehicle-treated animals were sampled randomly. Liver samples were immediately frozen in liquid nitrogen and stored at −70°C for subsequent measurement of ODC activity. The specimens were pooled and homogenized in 0.25 ml of homogenizing buffer containing 0.25 *M* sucrose, 50 m*M* Tris-HCl (pH 7.5), 1 m*M* EDTA, 0.4 m*M* pyridoxal 5′-phosphate, and 1 m*M* dithiothreitol in an Ultra-Turrax tissue homogenizer. They were then centrifuged at 15,000*g* for 30 min at 4°C. The supernatant was assayed for ODC activity by a modification of the micromethod of Lans *et al.*[11] in an

Eppendorf microfuge in a final volume of 40 μl. The reaction mixture (final concentration) contained 50 m*M* 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (pH 7.4), 1 m*M* EDTA, 0.25 m*M* pyridoxal 5′-phosphate, 1 m*M* dithiothreitol, and 130 μM [1-^{14}C]-ornithine (40.6 mCi/mmol; Amersham International Plc, Amersham, UK). The reaction at 37°C was initiated with 20 μl of the supernatant, and the liberated $^{14}CO_2$ was collected after 60 min incubation on paper filter in the top of microtubes with 10 μl of 10% potassium hydroxide, and then the reaction was terminated by adding 10 μl of 6 *N* hydrochloric acid. The sample was incubated for an additional 15 min to collect $^{14}CO_2$ completely. At the end of this period, the paper filter including $^{14}CO_2$ was removed, immersed in a scintillation vial, and counted for radioactivity in 10 ml of scintillation cocktail. One enzyme unit is defined as 1 pmol of $^{14}CO_2$ released/mg protein/h. Protein content was measured by use of a Bio-Rad protein assay kit (Bio-Rad, Richmond, CA), with bovine serum albumin as the standard.

Assay for antioxidant activity

Rabbit erythrocyte membrane ghost system: Commercially available rabbit blood (100 ml) was diluted with 300 ml of an isotonic buffer solution (10 m*M* phosphate buffer at pH 7.4/152 m*M* NaCl). The solution was centrifuged (3,500 rpm for 20 min), and the blood was lysed in 300 ml of a 10 m*M* phosphate buffer at pH 7.4. The erythrocyte membrane ghosts were pelleted by centrifugation (11,500 rpm for 40 min), and the precipitate was diluted to give a suspension (1.0 mg of protein/ml). Peroxidation of the erythrocyte membrane ghosts induced by t-BuOOH was carried out according to Osawa's method[12] in the prosence or absence of MMTS. After incubaton at 37°C for 20 min, the amount of thiobarbituric acid-reactive substances was determined at 532 nm.

Rat liver assay: Nine male F344 rats, 5 weeks of age, were divided into 3 groups. Rats in group 2 were given the diet containing 100 ppm MMTS from the start of experiment and animals in the other groups were given the basal diet. Animals in groups 1 and 2 were given i.p.-injections of 2-nitropropane (2NP) (100 mg/kg body weight) at 10 days after the start of experiment, and group 3 received a single i.p.-injection of saline (vehicle). Twenty-four hours after the injection, the rats were killed by decapitation under ether anesthesia, and the livers were promptly excised and chilled in ice-cold PBS. After having been washed with PBS, the specimens were homogenized in 1 ml of homogenizing buffer, 50 m*M* Tris-HCl (pH 7.4) in an Ultra-Turrax tissue homogenizer. The homogenate was centrifuged at 15,000*g* for 30 min at 4°C. The supernatant was assayed for lipid peroxidation activity. Peroxidation of the specimens was carried out according to Osawa's method.[12] Protein content was measured by use of a Bio-Rad protein assay kit (Bio-Rad), with bovine serum albumin as the standard.

Statistical analyses Differences in the incidence or density of pathological lesions in the liver between groups were analyzed by use of the χ^2-test, Fisher's exact probability test or Student's *t* test.

RESULTS

General observations There was no clear evidence of toxicity in animals exposed to the MMTS diet (Table I). Two rats in group 5 died of pneumonia before termination of the experiment, but no neoplasms were found in them. The MMTS diet reduced the body and liver weights compared to those in the other groups. Relative liver weight was significantly increased by PB and reduced by MMTS compared to the appropriate control.

Tumor incidence Liver tumors were only recognized in DEN-treated groups. The neoplasms were those of hepatocellular origin (Table II). The incidences of adenomas in groups 3 and 4 were significantly lower than that in group 1 ($P<0.005$ and $P<0.0001$, respectively). The incidences of total liver neoplasms of groups 3 and 4 were

Table I. Body and Liver Weights of Rats

Group	Treatment	No. of rats	Body weight (g)	Liver weight (g)	Relative liver weight (%)
1	DEN → PB	20	322.7±29.5[a]	15.9±1.9	4.96±0.42
2	MMTS+DEN → PB	21	317.8±28.2	15.1±2.0	4.77±0.51
3	DEN → MMTS+PB	20	310.5±20.9	14.3±1.6	4.63±0.48
4	DEN alone	24	325.8±18.8	11.7±1.2	3.59±0.47
5	DEN → MMTS	18	307.4±11.4	10.1±1.7	3.48±0.37
6	MMTS alone	8	336.5±22.3	10.9±1.8	3.21±0.40
7	PB alone	8	357.0±23.9	16.1±2.4	4.51±0.59
8	Vehicle control	14	351.6±13.8	11.9±0.9	3.39±0.24

a) Mean±SD.

Table II. Incidences of Liver Tumors in Rats Treated with DEN and/or MMTS

Group	Treatment	Incidence(%)			Multiplicity		
		Ad.[a]	Ca.[b]	total	Ad.	Ca.	total
1	DEN → PB	95	70	100	3.2±2.4[c]	2.0±1.9	5.2±3.9
2	MMTS+DEN → PB	89	89	100	4.3±3.8	2.8±1.6	7.2±4.8
3	DEN → MMTS+PB	55[d]	81	75[e]	1.5±2.1[f]	0.9±0.9[f]	2.5±2.4[g]
4	DEN alone	13	29	42	0.1±0.3	0.4±0.6	0.5±0.7
5	DEN → MMTS	18	24	41	0.2±0.4	0.2±0.4	0.4±0.5
6	MMTS alone	0	0	0	—	—	—
7	PB alone	0	0	0	—	—	—
8	Vehicle control	0	0	0	—	—	—

a) Hepatocellular adenoma.
b) Hepatocellular carcinoma.
c) Mean±SD.
d, e) Significantly different from group 1 by Fisher's exact probability test (*d*) $P<0.005$, *e*) $P<0.05$).
f, g) Significantly different from Group 1 by Student's *t* test (*f*) $P<0.05$, *g*) $P<0.002$).

Table III. Quantitative Analysis of GST-P-positive Foci in Rats Treated with DEN and/or MMTS

Group	Treatment	Density (/cm²)	Average area ($\times 10^{-2}$ cm²)	Unit area (%)
1	DEN → PB	46.6±7.6[a]	13.9±3.5	6.5±1.8
2	MMTS+DEN → PB	45.6±8.0	14.0±3.7	6.6±2.4
3	DEN → MMTS+PB	40.6±8.0[b]	11.7±2.6[c]	4.9±1.5[d]
4	DEN alone	21.6±6.3	9.4±3.1	2.1±1.0
5	DEN → MMTS	15.3±6.0[e]	9.3±2.0	1.5±0.8[f]

a) Mean±SD.
b–d) Significantly different from the rats treated with DEN → PB by Student's *t* test (*b*) $P<0.05$, *c*) $P<0.0001$, *d*) $P<0.01$).
e, f) Significantly different from the rats treated with DEN alone by Student's *t* test (*e*) $P<0.005$, *f*) $P<0.05$).

significantly reduced compared to group 1 ($P<0.05$ and $P<0.0001$, respectively). The incidence of hepatocellular carcinomas in group 4 was significantly lower than that of group 1 ($P<0.01$). No significant differences in the incidences could be found between groups 4 and 5. The incidence of hepatocellular adenomas in group 2 was lower than that of group 1, although the difference was not statistically significant. The incidence of hepatocellular carcinomas in group 3 was slightly increased compared to that of group 1, although again the difference was not significant. The multiplicities of adenomas, carcinomas and total tumors in groups 2 and 4 were significantly lower than those in group 1 ($P<0.05$, $P<0.05$ and $P<0.002$, and $P<0.0001$, $P<0.0005$, and $P<0.0001$, respectively). The multiplicities of adenomas, carcinomas and total tumors in group 3 were slightly higher than those of group 1, although the differences were not significant. No significant differences in the incidence and multiplicity of tumors were found between groups 4 and 5.

Incidence of hepatocellular foci In this study, three types of preneoplastic hepatocellular foci (clear, eosinophilic and basophilic) giving a positive GST-P reaction were found in all groups exposed to DEN. A few liver cell foci were also found in several animals in the vehicle-treated groups. The results of quantitative analysis of the frequency of GST-P-positive foci are summarized in Table III. The density, average area and unit area of GST-P-positive foci of group 1 were greater than those of groups 3 and 4. The density of GST-P-positive foci of group 5 was smaller than that of group 4. The average area and unit area of GST-P-positive foci of group 5 were smaller than those of group 4, although the differences were not significant (Table III).

Results of ODC assay Table IV summarizes the ODC activities in liver tissues without macroscopic tumors. The ODC activity in the liver of rats treated with DEN → PB (group 1) was significantly higher than those of rats given DEN → PB with MMTS in the initiation phase or promotion phase (groups 2 and 3) ($P<0.0001$

Table IV. ODC Activity of Rat Livers

Group	Treatment	No. of rats	ODC activity (pmol $^{14}CO_2$/mg protein/h)
1	DEN → PB	20	39.03±10.81[a]
2	MMTS+DEN → PB	21	9.37±2.37[b]
3	DEN → MMTS+PB	20	11.33±3.54[b]
4	DEN alone	24	30.93±17.91
5	DEN → MMTS	18	18.93±27.66
6	MMTS alone	8	6.99±8.75
7	PB alone	8	10.16±6.37
8	Vehicle control	14	4.74±1.27[c]

a) Mean±SD.
b) Significantly different from the rats treated with DEN → PB by Student's *t* test ($P<0.0001$).
c) Significantly different from the rats treated with PB alone by Student's *t* test ($P<0.01$).

in each). In this experiment, DEN or PB treatment alone significantly increased the ODC activity of the liver ($P<0.0001$ or $P<0.01$, respectively). Administration of MMTS in the post initiation phase (group 5) also reduced the ODC activity in the liver compared with DEN-alone treatment (group 4), although the difference was not significant (Table IV).

Results of antioxidant assay The modifying effect of MMTS on lipid peroxidation was measured in rabbit erythrocyte membrane ghosts, compared with a positive control and an untreated control. MMTS reduced lipid peroxidation to 77% of the positive control at 10^{-2} mol/liter.

The lipid peroxide level of rat liver treated with 2NP was 2.367±0.479 nmol malondialdehyde (MDA)/mg protein, whereas that of untreated control rat liver was 1.251±0.033 nmol MDA/mg protein. The lipid peroxide level of rat liver treated with MMTS and 2NP was 1.183 ±0.069 nmol MDA/mg protein. Thus, lipid peroxidation induced by 2NP was markedly decreased by MMTS.

DISCUSSION

The results of the present study indicate that MMTS has a suppressive effect on the promotion stage of DEN-initiated and PB-promoted hepatocarcinogenesis in rats. MMTS suppressed the occurrence of liver neoplasms more clearly after treatment with carcinogen and promoter than after carcinogen alone. The results of quantitative analysis of altered liver cell foci using a phenotypic marker, GST-P, were also in agreement with the above result. These liver cell foci are generally recognized as preneoplastic lesions and reflect carcinogenic potential due to the consistent manner in which liver cell foci appear during the post-initiation stage of hepatocarcinogenesis.[13] In studies of hepatocarcinogenesis, a variety of markers for altered foci, such as GST-P, γ-glutamyltranspeptidase (GGT), adenosine triphosphatase and glucose-6-phosphatase have been used, and GST-P, which can detect a single initiated cell, is regarded as the most reliable.[13-16]

In our current study, dietary exposure to MMTS during the initiation or promotion phase of DEN/PB-induced hepatocarcinogenesis decreased hepatic ODC activity in non-lesion areas. ODC induction is reported to precede cell proliferation in many cells exposed to xenobiotics, including genotoxic carcinogens[17]; the activity is considered to be a marker for cell proliferation,[18,19] which is an important event during carcinogenesis.[20,21] In this experiment, ODC activity was enhanced by PB treatment. This suggests a clear association between ODC activity and promoting action of PB in hepatocarcinogenesis. Cell proliferation enhances the frequency of tumor formation in many organs[22] including the liver.[23-27] In association with DNA adduct formation, liver cell proliferation clearly enhances the initiation of carcinogenesis.[23] Enhanced cell proliferation may be related to the fixation of mutagenic events.[23] Interestingly, in our current study, ODC activity was also enhanced by DEN treatment. Similar findings were reported by other investigators using different hepatocarcinogenesis models.[24-27] Thus, ODC activity may have an important role in the initiation process, and decrease of ODC activity is considered to be one of the mechanisms of the chemopreventive action of MMTS.

In general, a number of mechanisms may be involved in the suppressive effects of chemopreventive agents on cancers. Various compounds, especially those having antioxidative properties, are known to exert antimutagenic and anticarcinogenic activities.[28-31] Thus, antioxidant effect has been proposed as an important mechanism of the chemopreventive effect of MMTS. In this study, oral administration of MMTS suppressed lipid peroxidation induced by 2-NP. Also, MMTS mildly reduced lipid peroxidation in the erythrocyte membrane. In addition, oxygen free radicals are reported to be involved in the processes of multistage carcinogenesis, including the promotion phase.[32] Tumor-promoting agents, including PB, can induce oxygen radical formation by the hepatic mixed function oxidase system.[33-35] The inhibition of intercellular communication induced by PB is also reported to be prevented by antioxidants.[36] These results support the idea that antioxidants suppress the promotion activity of PB in hepatocarcinogenesis. It is suggested that MMTS decreases the free radicals in hepatocytes induced by PB.

Furthermore, Cu, Zn-superoxide dismutase is known to reduce DNA synthesis and mitosis of cultured neonatal rat hepatocytes induced by PB.[37] Antioxidants

have been reported to inhibit ODC induction and tumor promotion by a tumor promoter in skin carcinogenesis.[38] In this study, MMTS suppressed both ODC activity and lipid peroxidation. Accordingly, it is reasonable to conclude that there is a correlation between ODC activity and oxidative free radical induction in hepatocarcinogenesis.

Recently, Reddy *et al.*[8] provided evidence for chemopreventive effects of organosulfur compounds in colon carcinogenesis. They found that organosulfur compounds increase GST and NAD(P)H-dependent quinone reductase (NAD(P)H : QR) in the liver. The protective effects of these organosulfur compounds may be accounted for, at least in part, by their ability to induce GST and other phase II enzymes that are involved in carcinogen detoxification. In this context, further studies are needed to establish whether MMTS modulates phase I and phase II enzymes.

In conclusion, the results of the present investigation suggest that MMTS is a promising chemopreventive agent for human liver neoplasms.

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