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Lack of Chemoprevention Effects of the Monoterpene *d*-Limonene in a Rat Multi-organ Carcinogenesis Model

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Modifying effects of dietary administration of the monoterpene *d*-limonene were examined using a multi-organ carcinogenesis model. Groups of twenty F344 male rats were treated sequentially with *N*-diethylnitrosamine (DEN, i.p.), *N*-methyl-*N*-nitrosourea (MNU, i.p.), 1,2-dimethylhydrazine (DMH, s.c.), *N*-butyl-*N*-(4-hydroxybutyl)nitrosamine (BBN, in drinking water) and dihydroxy-di-*N*-propylnitrosamine (DHPN, in drinking water) during the first 4 weeks (DMBDD treatment), and then *d*-limonene was administered in the diet, at the dose of 2.0, 1.0 or 0.5%. The maximal tolerable dose was 2.0% under the present conditions. Further groups were treated with DMBDD or 2.0% *d*-limonene alone as controls. All surviving animals were killed at week 28, and major organs were examined histopathologically for development of preneoplastic and neoplastic lesions. The incidences and/or multiplicities of renal atypical tubules and adenomas were increased in animals fed 2.0% *d*-limonene. The immunohistochemical reactivity for α_{2u}-globulin in the proximal tubules was greater in rats fed *d*-limonene than in the carcinogen alone group. No enhancing or inhibitory effect was noted for tumor development in other organs. The present results indicate a lack of any chemopreventive effect of *d*-limonene in any organ of male rats under the present experimental conditions.

Key words: Modification of carcinogenesis — Multi-organ carcinogenesis — Chemoprevention — *d*-Limonene — α_{2u}-Globulin nephropathy

The monoterpene *d*-limonene is present in orange peel and essential oils of other plants. There have been many studies indicating chemopreventive efficacy against chemically induced carcinogenesis in various organs in rodents.[1-8] In chemically induced rat mammary tumor models, *d*-limonene was shown to possess preventive activity against both initiation and promotion.[2-6] It has been found to reduce the incidence of *N*-ethyl-*N*-hydroxyethylnitrosamine-induced liver tumors when given in the promotion stage to rats[9] and in mouse studies, it reduced *N*-diethylnitrosamine (DEN)-induced[7] and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone-induced[8] forestomach tumor and pulmonary adenoma formation when administered during the initiation phase.

On the other hand, in a 2-year carcinogenesis bioassay, *d*-limonene was found to induce kidney tumors in male rats, but not in females.[10] The renal tumor development was concluded to be associated with the α_{2u}-globulin nephropathy which occurs only in male rats given this chemical.[10] It was also shown that 30-day consecutive treatment with *d*-limonene by oral gavage caused a significant increase in the appearance of atypical tubules and atypical tubular hyperplasia in F344 male rats.[9]

In the present study, we investigated the potential post-initiation-phase chemopreventive activity of *d*-limonene at the whole organ level using a rat multi-organ carcinogenesis model developed in our laboratory.[11-15]

MATERIALS AND METHODS

Chemicals DEN, *N*-butyl-*N*-(4-hydroxybutyl)nitrosamine (BBN), and 1,2-dimethylhydrazine (DMH) were purchased from Tokyo Kasei Kogyo Co., Ltd. (Tokyo). *N*-Methyl-*N*-nitrosourea (MNU) was from Sigma Chemical Co. (St. Louis, MO). Dihydroxy-di-*N*-propylnitrosamine (DHPN) was from Nacalai Tesque Inc. (Kyoto). *d*-Limonene was from Wako Pure Chemical Industries, Ltd. (Osaka).

Animals A total of 100 male F344 rats, aged 5 week, was obtained from Charles River Japan Inc. (Kanagawa). They were randomly divided into five groups of 20 animals each and housed five to a plastic cage with hard chips for bedding in an air-conditioned animal room at 24±2°C and 55±5% humidity with a 12 h light/dark cycle. They were maintained on Oriental MF basal diet (Oriental Yeast Co., Tokyo) and tap water *ad libitum*.

Treatment The experimental design is presented in Fig. 1. Animals in groups 1–4 were treated sequentially with DEN (100 mg/kg body wt., i.p., single dose at commencement), MNU (20 mg/kg body wt., i.p., 4 times during weeks 1 and 2), BBN (0.05% in the drinking water during weeks 1 and 2), DMH (40 mg/kg body wt., s.c., 4 times during weeks 3 and 4), and DHPN (0.1% in the drinking water during weeks 3 and 4). This initial carcinogen treatment schedule (DMBDD treatment) was described in our previous papers.[11-15] From the end of week 4, groups 1–3 were respectively fed 2.0%, 1.0%

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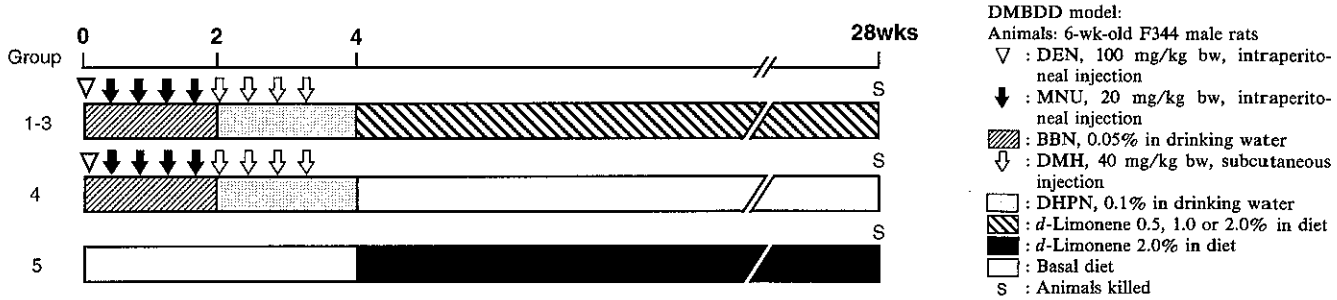

Fig. 1. Experimental protocol for the medium-term multi-organ carcinogenesis bioassay.

and 0.5% *d*-limonene in the basal diet for 24 week. The diets containing *d*-limonene were freshly prepared every 2 week. Group 4 received basal diet without test chemical supplement as a control group. Group 5 was fed 2.0% *d*-limonene in the basal diet for 24 week without prior DMBDD treatment. The dose of 2.0% had previously been established to be carcinogenic in a 2-year experiment.[10] Animals were weighed once a week for 6 week after the cessation of the initial carcinogen exposure, and then once every 4 week until the completion of the experiment. Animals surviving more than 22 week, when the first tumors appeared were included in the effective numbers. At week 28, all surviving animals were killed by exsanguination under ether anesthesia and subjected to complete necropsy.

Histopathological examination At necropsy, the livers and kidneys were weighed, and the relative percentage organ weights were calculated on the basis of final body weights. The major organs were excised, fixed in 10% buffered formalin, and routinely processed, and paraffin-embedded sections were stained with hematoxylin and eosin for histopathological examination. Liver slices fixed in ice-cold acetone were also prepared for quantitative assessment of immunohistochemically demonstrated glutathione S-transferase placental form (GST-P)-positive foci, as previously described.[16] GST-P-positive foci larger than 0.2 mm in diameter were analyzed with the aid of a color image processor (VIP-21C, Olympus-Ikegami Tsushin Co., Tokyo), and the data were expressed as numbers and areas/cm^2 liver tissue as previously described.[17] In addition, the kidneys were immunohistochemically investigated for α_{2u}-globulin and albumin accumulation (antibodies from Organon Teknika Co., West Chester, PA) using the ABC method (Vectastain ABC kit, PK4001, Vector Laboratories Inc., Burlingame, CA).

Statistical analysis In statistical analysis of differences between the treated and control groups, the criterion of significance was set at $P<0.05$. One way analysis of variance followed by Scheffe's multiple comparison test was applied to parametric data obtained for body weights, organ weights and GST-P-positive foci. The significance of differences in the incidences of proliferative lesions was evaluated using the χ^2 and cumulative χ^2 tests.

RESULTS

No clinical signs related to the test chemical treatment were apparent in any of the rats during the experiment. Three rats were found dead: one in group 1 at week 24, one in group 2 at week 27 and one in group 4 at week 22. The deaths were considered to have been caused by the DMBDD treatment in all cases. A slight reduction in final body weights was noted in rats of groups 1 and 2 compared to group 4, but this was not significant in the group 2 case (Table I). A significant increase in absolute and relative liver weights was noted in group 1 and a slight increase in relative liver weights was noted in group 2. A slight increase in absolute and relative kidney weights, due to renal tumors, was noted in group 2.

The incidence of kidney adenomas was significantly increased in group 1 (Table II) along with the average numbers of both atypical tubules and adenomas (Table III). However, no neoplastic renal lesions were observed in rats fed 2.0% *d*-limonene in the diet without prior DMBDD treatment. In rats fed *d*-limonene, numbers of hyaline droplets accumulated in the epithelial cells, with cytoplasmic basophilia and thickening of tubular basement membranes. Immunohistochemical examination revealed far stronger anti-α_{2u}-globulin reactivity in renal proximal tubules of rats fed *d*-limonene (groups 1, 2, 3 and 5) than in the controls (group 4).

Quantitative results for the numbers and areas of preneoplastic lesions, GST-P-positive foci, per cm^2 of liver tissue are shown in Table IV. A slight increase of the numbers and areas of GST-P-positive foci was noted in group 1 as compared to group 4, but the differences were not significant.

Table I. Final Body and Organ Weights

Group	Treatment	Effective no. of rats	Body wt.[a] (g)	Liver wt.[a] (g)	Liver wt.[a] (% b.w.)	Kidney wt.[a] (g)	Kidney wt.[a] (% b.w.)
1	DMBDD→ 2.0% *d*-limonene	19	317±12[b]	10.51±0.76[c]	3.32±0.18[c]	2.09±0.13	0.66±0.03
2	DMBDD→ 1.0% *d*-limonene	19	318±31	9.94±0.85	3.16±0.45[b]	2.38±0.88	0.75±0.23
3	DMBDD→ 0.5% *d*-limonene	20	324±18	9.40±0.24	2.90±0.24	2.18±0.36	0.67±0.14
4	DMBDD→ basal diet	19	336±27	9.68±1.02	2.88±0.14	2.14±0.25	0.64±0.07
5	None → 2.0% *d*-limonene	20	371±13	12.51±0.90	3.37±0.15	2.44±0.10	0.66±0.04

a) Mean±SD.
b, *c*) Significantly different from group 4 at $P<0.05$ and 0.01, respectively.

Table II. Incidences of Preneoplastic and Neoplastic Lesions of the Kidney

Group	Treatment	Effective no. of rats	Atypical tubules	Adenoma	Carcinoma	Nephroblastoma	TCC[c]
1	DMBDD→ 2.0% *d*-limonene	19	19 (100)[a]	13 (68)[b]	0	11 (58)	0
2	DMBDD→ 1.0% *d*-limonene	19	19 (100)	5 (26)	0	14 (74)	0
3	DMBDD→ 0.5% *d*-limonene	20	20 (100)	6 (30)	2 (10)	13 (65)	2 (10)
4	DMBDD→ basal diet	19	19 (100)	4 (21)	0	14 (74)	0
5	None → 2.0% *d*-limonene	20	1 (5)	0	0	0	0

a) Percentages in parentheses.
b) Significantly different from group 4 at $P<0.01$.
c) Transitional cell carcinoma.

Table III. Multiplicity Data for Atypical Tubules and Adenomas and Grade of α_{2u}-Globulin Staining in the Kidney

Group	Treatment	Effective no. of rats	Atypical tubules (No./rat)[a]	Adenoma (No./rat)[a]	α_{2u}-Globulin
1	DMBDD→ 2.0% *d*-limonene	19	9.26±2.60[b]	1.00±0.94[b]	±——+[c]
2	DMBDD→ 1.0% *d*-limonene	19	6.05±2.46	0.26±0.45	±——+
3	DMBDD→ 0.5% *d*-limonene	20	5.65±2.60	0.35±0.59	±
4	DMBDD→ basal diet	19	5.90±2.66	0.21±0.42	−——±
5	None → 2.0% *d*-limonene	20	0.05±0.22	0±0	±——+

a) Mean±SD.
b) Significantly different from group 4 at $P<0.01$.
c) Staining intensity: −, negative; ±, weakly positive; +, positive.

Table IV. Quantitative Data for GST-P-positive Liver Foci

Group	Treatment	Effective no. of rats	GST-P-positive liver foci[a] Numbers (No./cm²)	GST-P-positive liver foci[a] Areas (mm²/cm²)
1	DMBDD→ 2.0% *d*-limonene	19	10.8±3.2	1.15±0.34
2	DMBDD→ 1.0% *d*-limonene	19	9.2±3.8	0.92±0.35
3	DMBDD→ 0.5% *d*-limonene	20	7.4+3.3	0.78±0.39
4	DMBDD→ basal diet	19	7.7±2.1	0.87±0.34
5	None → 2.0% *d*-limonene	20	0±0	0±0

a) Mean±SD.

Table V. Incidences of Preneoplastic and Neoplastic Lesions in Other Organs

Organs and lesions	Group	1	2	3	4
	Dose (%)	2.0	1.0	0.5	0
	n	20	20	20	20
Lung					
Hyperplasia		20 (100)[a]	20 (100)	20 (100)	20 (100)
Adenoma		2 (10)	6 (30)	1 (5)	3 (15)
Carcinoma		0	1 (5)	0	1 (5)
Tongue					
Papilloma		0	0	0	1 (5)
Esophagus					
Hyperplasia		8 (40)	9 (45)	8 (40)	11 (55)
Forestomach					
Hyperplasia		3 (15)	4 (20)	6 (30)	6 (30)
Small intestine					
Adenoma		4 (20)	3 (15)	3 (15)	8 (40)
Carcinoma		3 (15)	9 (45)	7 (35)	2 (10)
Large intestine					
Adenoma		6 (30)	3 (15)	5 (25)	2 (10)
Carcinoma		4 (20)	7 (35)	0	2 (10)
Urinary bladder					
PN Hyperplasia[b]		0	2 (10)	0	4 (20)
Papilloma		4 (20)	3 (15)	3 (15)	2 (10)
Carcinoma		1 (5)	3 (15)	0	1 (5)
Thyroid gland					
Hyperplasia		3 (15)	2 (10)	2 (10)	0
Adenoma		1 (5)	4 (20)	3 (15)	1 (5)
Carcinoma		1 (5)	0	1 (5)	2 (10)
Spleen					
Hemangioma		0	1 (5)	0	0

a) Percentages in parentheses.
b) Papillary or nodular hyperplasia.

Data for histopathological findings in organs other than the kidney and liver are summarized in Table V. Although hyperplastic or neoplastic lesions were observed in many organs, such as the lung, thyroid, urinary bladder and small and large intestines, no modification of carcinogenesis in these organs by *d*-limonene was found.

DISCUSSION

The present investigation of the chemopreventive potential of *d*-limonene in a rat multi-organ carcinogenesis model demonstrated clear enhancement of the development of kidney preneoplastic and neoplastic lesions, but no inhibiting effect on carcinogenesis in any organ. The accumulation of α_{2u}-globulin in the renal proximal tubules of rats given *d*-limonene which was immunohistochemically demonstrated in the present study is in agreement with observations by Dietrich *et al.*,[9] who reported that *d*-limonene promotes preneoplastic lesions and renal tumors only in the presence of the male rat-specific urinary protein, α_{2u}-globulin. α_{2u}-Globulin-chemical complexing leads to an accumulation of this protein in the proximal tubules of the male rat kidney and to morphological changes.[18–25]

d-Limonene was earlier demonstrated to regress completely rat mammary carcinomas induced by 7,12-dimethylbenz(*a*)anthracene or MNU.[5] This regression was only achieved at a dose of 7.5% in diet and no effect was observed with a dose of 5.0%. The dose of 7.5% is extremely high and was not tolerated by male rats in a preliminary experiment in our laboratory because of its nephrotoxicity due to the accumulation of α_{2u}-globulin. That is the reason why 2.0% was chosen as the maximal dose in the present experiment.

d-Limonene is also known to inhibit DNA-adduct formation in the liver, lung, spleen and kidney and to increase the levels of members of the cytochrome P450 2B and 2C families in female Wistar rats.[6] Inhibition of forestomach and pulmonary tumors using A/J mice was observed when *d*-limonene was given in the initiation stage. Kawamori *et al.* recently reported inhibitory effects of *d*-limonene on the development of aberrant crypt foci in rat colon.[26] All reported chemopreventive effects, except for the above-mentioned inhibition of

mammary carcinoma development, were observed when *d*-limonene was given with carcinogen treatment or in the initiation stage. Thus, it can be considered that *d*-limonene may inhibit the action of carcinogens in the target organ cells by enhancing the detoxification system. Although the present results would indicate that its post-initiation chemopreventive potential is limited, this does not necessarily detract from the efficacy of *d*-limonene as a chemopreventive, since no obvious adverse effects were noted.

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