

Inhibition of *N*-Nitrosodiethylamine Carcinogenesis in Mice by Naturally Occurring Organosulfur Compounds and Monoterpenes¹

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ABSTRACT

Naturally occurring compounds belonging to two chemical groups were studied for their capacities to inhibit *N*-nitrosodiethylamine (NDEA)-induced carcinogenesis in female A/J mice. One group consists of organosulfur compounds found in *Allium* species, including garlic, onions, leeks, and shallots, and the other, two monoterpenes, *i.e.*, D-limonene and D-carvone. In an initial experiment, in which organosulfur compounds were investigated, diallyl disulfide, allyl mercaptan, and allyl methyl disulfide were found to produce a marked inhibition of NDEA-induced neoplasia of the forestomach when the test compounds were administered *p.o.* 96 and 48 h prior to NDEA. The most potent was diallyl disulfide which reduced forestomach tumor formation by more than 90%. Pulmonary adenoma formation also was inhibited but to a considerably lesser extent, *i.e.*, about 30%. In three additional experiments, test compounds were given *p.o.* either 15 min or 1 h prior to NDEA. Under these conditions diallyl disulfide and allyl mercaptan again inhibited forestomach tumor formation substantially, *i.e.*, greater than 75%, and pulmonary adenoma formation marginally, *i.e.*, less than 20%. In these experiments D-limonene and D-carvone were tested and reduced forestomach tumor formation by slightly over 60% and pulmonary adenoma formation by about 35%. The results of these studies provide evidence of an increasing diversity of naturally occurring compounds having the capacity to inhibit nitrosamine carcinogenesis.

INTRODUCTION

Naturally occurring compounds belonging to two chemical groups were studied for their capacities to inhibit NDEA³-induced carcinogenesis. One group consisted of organosulfur compounds found in *Allium* species, including garlic, onions, leeks, and shallots (1-6). The other contained two monoterpenes, *i.e.*, D-limonene and D-carvone. D-Limonene is a constituent of citrus fruits (7, 8). D-Carvone occurs in caraway seeds (9).

Garlic and onions have been cultivated since antiquity and have been used as foodstuffs and medicines. A very extensive literature exists concerning medicinal uses and chemical composition of these plants (10-12). Among the biological effects found is prevention of cancer. Garlic and onion oils and pure organosulfides obtained from these plants have been shown to inhibit carcinogenesis (13-19). Inhibition of the occurrence of neoplasia has been obtained by using three experimental formats. In the first, the test compound was administered 96 and 48 h prior to the carcinogen. Under these conditions allyl methyl trisulfide, AMD, DAS, and diallyl trisulfide given by *p.o.* intubation were found to inhibit BP-induced neoplasia of the forestomach in female A/J mice (15, 17). Inhibition studies with other carcinogens using these experimental conditions have not been published. In the second format, the test compound was administered by gavage 3 h prior to the carcinogen.

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³ The abbreviations used are: NDEA, *N*-nitrosodiethylamine; AMD, allyl methyl disulfide; DAS, diallyl sulfide; BP, benzo(*a*)pyrene; AM, allyl mercaptan; DADS, diallyl disulfide.

With this temporal sequence, 1,2-dimethylhydrazine-induced neoplasia of the large bowel in mice and *N*-nitrosomethylbenzylamine-induced esophageal carcinogenesis in the rat were found to be inhibited by DAS (16, 19). The third format entailed inhibition of tumor promotion. For this purpose garlic oil, ajoene or 1-propenyl sulfide were applied to the skin of mice 30 min after topical application of phorbol-myristate acetate (13, 14). In these latter experiments inhibition of lipoxygenase and ornithine decarboxylase was observed.

The present studies entail a continuation of the previous work with the organosulfur compounds (15, 17). The chemical structures of the compounds studied are shown in Fig. 1. The format used in obtaining inhibition of BP carcinogenesis has been applied to the investigations of NDEA carcinogenesis. The test compounds were administered 96 and 48 h prior to the carcinogen challenge. In addition, several organosulfur compounds were studied in experiments in which the test compounds were given 15-60 min prior to carcinogenic challenge. Based on the work of Wargovich *et al.* (16, 19) and Brady *et al.* (20), it appeared likely that inhibition might be obtained under these conditions. A preliminary report of some of the experiments has been presented elsewhere (18).

Two monoterpenes, D-limonene and D-carvone have been included in the present investigations (Fig. 1). Their selection was based on the fact that they both contain an allylic substituent. In previous studies of inhibition of BP carcinogenesis by organosulfur compounds, the structure-activity relationships indicated that allyl groups were important to the inhibitory effects observed (17). Accordingly, it appeared useful to study allyl groups present in a structure differing from the organosulfur compounds. Monoterpenes are a particularly interesting group of compounds. They occur widely in foods consumed by humans (7-9). In addition, a considerable amount of work has been done on the inhibitory capacities of D-limonene and citrus fruit oils (21-23). In experiments in which orange oil was fed in the diet, inhibition of BP-induced neoplasia of the forestomach and lung occurred, as well as inhibition of 7,12-dimethylbenz(*a*)anthracene-induced mammary tumor formation (22, 23). Orange oil contains over 90% D-limonene. In other work in which pure D-limonene was used, inhibition of 7,12-dimethylbenz(*a*)anthracene-induced carcinogenesis was found (21). Thus, monoterpenes appear to be a promising group for study as potential chemopreventive agents.

MATERIALS AND METHODS

Chemicals. AMD was synthesized as described previously, purity approximately 98% (17); DADS, Aldrich Chemical Co., Milwaukee, WI was purified by vacuum distillation, purity >98%; and AM, Fairchild Chemical Co., Blythewood, SC, or Aldrich Chemical Co., was freshly distilled before use; boiling point 67.0-67.5°C. The following compounds were of commercial origin (Aldrich Chemical Co.) and were used without further purification: DAS, 97%; dipropyl disulfide, 97%, and D-carvone, 96%. D-Limonene, 99%, and NDEA were purchased from the Sigma Chemical Co., St. Louis, MO, and caraway seed oil was from Lorann Oils, Lansing, MI.

Animal Experiments. Female A/J mice from The Jackson Labora-

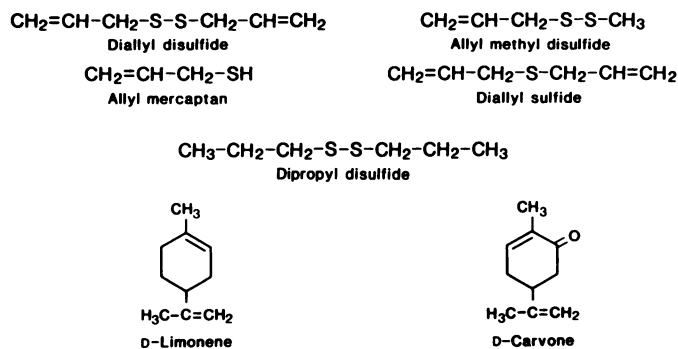


Fig. 1. Chemical structures of compounds studied.

tory, Bar Harbor, ME, were used in all experiments. Mice were randomized by weight at 8 weeks of age into groups of 15 or 16 mice. They were placed on a semipurified diet consisting of 27% vitamin-free casein, 59% starch, 10% corn oil, 4% salt mix (USP XIV), and a complete mixture of vitamins (Teklad, Madison, WI). In the first experiment, the mice were given two administrations of the test compound or the vehicle (cottonseed oil) by p.o. intubation 48 h apart starting 1 week after having been placed on the semipurified diet; 48 h after the second administration they were given NDEA, 20 mg/kg body weight in 0.2 ml H₂O by p.o. intubation. This sequence of administrations was repeated once a week for a total of eight times. The mice remained on the semipurified diet until 3 days after the last administration of NDEA. Then they were fed Purina rat chow (Ralston Purina, St. Louis, MO) and were maintained on this diet for the duration of the protocol. Mice were weighed at intervals of 4 weeks. The experiments were terminated 26 weeks after the initial dose of carcinogen, at which time the mice were autopsied. The stomachs were removed and formalin was injected into them so as to be fixed in an expanded state. Tumors of the forestomach and pulmonary adenomas were counted as described previously (17, 24–26). Histopathology examinations were performed to ascertain the nature of the tumors. In the second set of experiments in which a short time interval between administration of test compound and NDEA was studied, the same conditions were used as described above except that the test compound or vehicle was administered either 15 min or 1 h prior to NDEA.

Statistical Analysis. Student's *t* test was used to determine the significance of the differences in the number of pulmonary adenomas per mouse between the control and treated groups. The *U*-test of Wilcoxon, Mann, and Whitney was used to determine the significance of the differences in the number of papillomas per mouse and the χ^2 test with Yates' correction or Fisher's exact test were used for the differences in the percentage of tumor-bearing animals in these groups. All tests were two-sided.

RESULTS

The data obtained from Experiment 1 show that the three organosulfur compounds tested inhibit carcinogenesis of the forestomach of the female A/J mice when given 96 and 48 h prior to the NDEA (Table 1). The inhibition was manifested by a reduction in the number of papillomas present and the lack of the occurrence of carcinomas under the conditions of the experiment. The greatest inhibitory effect was obtained with DADS. It resulted in more than 90% reduction of the mean number of papillomas/mouse. In contrast, only about 30% reduction of the occurrence of pulmonary adenomas by the three compounds was observed. In Experiment 2 the same compounds were studied for their capacity to inhibit neoplasia when administered 15 min before NDEA. A different ranking order of inhibitory effects was found. In the forestomach AM was the most potent inhibitor. It gave more than 88% reduction in the mean number of papillomas/mouse. DADS resulted in more than 77% reduction of papillomas/mouse whereas AMD

and DAS were almost inactive. The compounds produced only small or no significant reduction of pulmonary adenoma formation.

Experiments 3 and 4 differ from Experiment 2 in that the test compounds were given 1 h prior to NDEA. As in Experiment 2, DADS was a potent inhibitor of forestomach tumor formation. In this case it lowered the mean number of papillomas/mouse by more than 97%. DAS was almost inactive. Neither compound inhibited pulmonary adenoma formation. In Experiment 3, dipropyl disulfide, the saturated counterpart to DADS, was tested and found to have some inhibitory activity in the forestomach. Its use resulted in more than 60% reduction in the mean number of papillomas. Formation of carcinomas were also reduced by these compounds. Activity paralleled the reduction of papillomas.

Two monoterpenes have been investigated for their chemopreventive effects. In Experiment 3, D-carvone was found to inhibit forestomach tumor formation. More than 63% reduction in the mean number of papillomas occurred. It also inhibited pulmonary adenoma formation, but to a lesser extent, *i.e.*, 34%. Similar inhibitory properties were shown by caraway seed oil. This oil contains about 50% D-carvone and, in addition, a variety of related monoterpenes (9). In Experiment 4, D-limonene was found to inhibit NDEA carcinogenesis by more than 97%.

DISCUSSION

In previous investigations the effects of organosulfur compounds in garlic and onions were studied for their inhibitory effects on BP-induced neoplasia of the forestomach and lung of female A/J mice when administered 96 and 48 h prior to carcinogen challenge (15, 17). The results of those studies indicated that the inhibitory capacities of the compounds were largely dependent upon the presence of allyl groups. Compounds containing one or more allyl groups inhibited, whereas their saturated analogues were almost without activity. Compounds containing two allyl groups were more potent than those containing one allyl group. In Experiment 1 of the present study in which the test compounds were administered 96 and 48 h prior to NDEA, the importance of the allyl group is again evident. Thus, the most potent of the three organosulfur compounds studied was diallyl disulfide. Allyl methyl disulfide, which contains only one allyl group, was less potent. Allyl mercaptan which was used at one-half the molar concentration of diallyl disulfide and allyl methyl disulfide had an inhibitory activity comparable with the latter compound. This activity would be in accord with content of the allyl groups contributed by the two compounds. As in the case of BP carcinogenesis, the inhibitory effects on NDEA-induced forestomach neoplasia were of a much greater magnitude than those on pulmonary adenoma formation.

The 96- and 48-h administration schedule was originally chosen because of studies demonstrating that organosulfur compounds induce increased glutathione *S*-transferase activity under these conditions (15, 17). Compounds that induce an increase in activity of one Phase II enzyme such as glutathione *S*-transferase frequently induce increases in activity of other Phase II enzymes as well as glutathione concentration. Thus, one could speculate that these inductive effects were at least partly responsible for the inhibitions observed in Experiment 1.

In Experiments 2–4, the test compounds were administered either 15 min or 1 h prior to NDEA. The short time interval

Table 1 Effects of test materials on NDEA-induced neoplasia in female A/J mice

Cottonseed oil (0.2 ml) or test material in 0.2 ml cottonseed oil were administered by p.o. intubation once a week for 8 weeks to female A/J mice at the time interval designated prior to NDEA, 20 mg/kg body weight. The experimental treatment was started when the mice were 9 weeks old; the experiment was terminated 26 weeks after the first dose of NDEA.

Experiment	Pretreatment	Time interval prior to NDEA	No. of mice at risk ^a	Forestomach tumors				Pulmonary adenomas (no. of tumors/mouse) ^f	Wt gain from 8 to 35 weeks of age (g)
				% of mice with papillomas	% of mice with >30 papillomas ^b	Median no. of papillomas	% of mice with carcinomas		
1	None	96 and 48 h	13	100	100	>30	31	14.2 ± 1.3 ^d	7.6
	Cottonseed oil		15	100	100	>30	20	14.1 ± 1.3	7.3
	Diallyl disulfide (0.02 mmol)		13	100	0 ^e	3.0 ^e	0 ^f	9.9 ± 1.2 ^e	7.0
	Allyl mercaptan (0.01 mmol)		14	100	57 ^e	>30	0 ^f	9.6 ± 1.3 ^e	7.1
	Allyl methyl disulfide (0.02 mmol)		15	100	60 ^e	>30	0 ^h	9.3 ± 0.8 ⁱ	6.9
2	None	15 min	14	100	100	>30	29	15.9 ± 1.0	5.9
	Cottonseed oil		13	100	100	>30	38	16.6 ± 0.8	7.0
	Diallyl disulfide (0.02 mmol)		14	100	0 ^e	7.0 ^e	7	13.6 ± 1.1 ^j	5.6
	Diallyl sulfide (0.02 mmol)		14	100	100	>30	21	12.0 ± 0.9 ⁱ	5.6
	Allyl mercaptan (0.01 mmol)		12	92	0 ^e	3.5 ^e	0 ^f	14.2 ± 1.6	6.8
	Allyl methyl disulfide (0.02 mmol)		15	100	73	>30	27	12.5 ± 1.0 ^e	7.3
3	None	1 h	16	100	100	>30	25	15.4 ± 1.3	7.5
	Cottonseed oil		15	100	100	>30	20	13.3 ± 0.9	7.0
	D-Carvone (0.2 mmol)		14	100	0 ^e	11.0 ^e	0 ^f	8.8 ± 0.9 ⁱ	5.5
	Diallyl sulfide (0.02 mmol)		15	100	100	>30	13	16.3 ± 1.8	5.3
	Dipropyl disulfide (0.02 mmol)		13	100	0 ^e	12.0 ^e	8	11.5 ± 1.0	6.5
	Caraway seed oil (25 μl)		15	100	0 ^e	3.0 ^e	0 ^h	8.6 ± 0.7 ^e	6.7
4	Cottonseed oil	1 h	15	100	73	>30	27	10.4 ± 1.4	5.3
	Diallyl disulfide (0.02 mmol)		12	58 ^j	0 ^k	1.0 ^k	0 ^f	10.8 ± 1.0	4.9
	D-Limonene (0.2 mmol)		15	67 ^j	0 ^k	1.0 ^k	0 ^h	6.5 ± 0.6 ^j	4.2

^a Mice surviving duration of protocol.

^b The number of papillomas per forestomach beyond 30 was not counted. Further counting is not accurate because of fusion of lesions.

^c Number of tumors in the entire group/number of mice at risk.

^d Mean ± SE.

^e $P < 0.001$ versus either vehicle or absolute control.

^f $P = 0.05$ versus vehicle controls combined from all four experiments.

^g $P < 0.05$ versus either vehicle or absolute control.

^h $P < 0.05$ versus vehicle controls combined from all four experiments.

ⁱ $P < 0.01$ versus either vehicle or absolute control.

^j $P < 0.05$ versus vehicle control.

^k $P < 0.001$ versus vehicle control.

was chosen on the basis of findings by Wargovich *et al.* (16, 19) showing that DAS inhibits *N*-nitrosobenzylamine-induced esophageal cancer in rats when given 3 h prior to the carcinogen. The short time interval suggested that the inhibitory effects may have been due to inhibition of carcinogen activation. In a recent study, evidence for such enzyme inhibition has been presented (20). The data obtained in Experiments 2 and 3 are complicated in terms of structure-activity relationships. The allyl group, again, appears important, but the inhibition obtained with dipropyl disulfide suggests that saturated compounds also may inhibit. DADS and AM showed potent inhibitory effects. AMD and DAS were almost inactive. The pattern of inhibitory effects is different from that found in Experiment 1. This is evident by the relationships between inhibition in the forestomach by AM and AMD in the two experiments. The findings suggest that liberation of AM from disulfides may be of importance to the occurrence of inhibition. Such liberation could result from splitting of disulfides. The near absence of inhibitory effect of DAS in the present study could be accounted for by inability of the compound to be converted to AM under the experimental conditions used. However, DAS given 3 h prior to *N*-nitrosomethylbenzylamine or dimethylhydrazine, produces profound inhibitory effects (16, 19). Several explanations exist for the lack of inhibition by DAS in the present study and the marked inhibition observed in those previous studies. They include differences in carcinogen, the longer time interval between test compound and carcinogen administration, and differences in animal species used. However, definitive data remain to be obtained.

D-Carvone and D-limonene were originally chosen for study

on the basis of containing an allyl substituent, since the data from the experiments with the organosulfur compounds indicated the importance of this functional group in chemoprevention of carcinogenesis. Both compounds produced pronounced inhibition of forestomach tumor formation and, in addition, significant though smaller inhibition of pulmonary adenoma formation. The results of the present study provide evidence for an increasing diversity of naturally occurring compounds having the capacity to inhibit nitrosoamine carcinogenesis (27). The impact of such inhibitory effects on environmental exposure of human populations to this class of carcinogens could be of importance, but clear evidence for such inhibitory effects remains to be demonstrated.

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