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Citrus Auraptene Exerts Dose-dependent Chemopreventive Activity in Rat Large Bowel Tumorigenesis: The Inhibition Correlates with Suppression of Cell Proliferation and Lipid Peroxidation and with Induction of Phase II Drug-metabolizing Enzymes[1]

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ABSTRACT

In our previous short-term experiment, *Citrus* auraptene inhibited the development of azoxymethane (AOM)-induced aberrant crypt foci, which are precursor lesions for colorectal carcinoma. In the present study, the possible inhibitory effect of dietary administration of auraptene was investigated using an animal colon carcinogenesis model with a colon carcinogen AOM. Male F344 rats were given s.c. injections of AOM (15 mg/kg body weight) once a week for 3 weeks to induce colon neoplasms. They also received diets containing 100 or 500 ppm auraptene for 4 weeks in groups of "initiation" feeding, starting 1 week before the first dosing of AOM. The diets containing auraptene were also given to rats for 38 weeks in groups of "postinitiation" feeding. At the termination of the study (38 weeks), dietary administration of auraptene caused dose-dependent inhibition in AOM-induced large bowel carcinogenesis. Auraptene feeding during the initiation phase reduced the incidence of colon adenocarcinoma by 49% at 100 ppm ($P = 0.099$) and 65% at 500 ppm ($P = 0.0075$). Auraptene administration during the postinitiation phase inhibited the incidence of colon adenocarcinoma by 58% at 100 ppm ($P = 0.021$) and 65% at 500 ppm ($P = 0.0075$). Also, the multiplicity of colon carcinoma was significantly reduced by initiation feeding at a dose level of 500 ppm ($P < 0.01$) and postinitiation feeding at a level of 100 and 500 ppm ($P < 0.05$ and $P < 0.01$, respectively). Feeding of auraptene suppressed the expression of cell proliferation biomarkers (ornithine decarboxylase activity and polyamine content) in the colonic mucosa and reduced the production of aldehydic lipid peroxidation [malondialdehyde and 4-hydroxy-2(*E*)-nonenal]. In addition, auraptene increased the activities of Phase II drug-metabolizing enzymes (glutathione *S*-transferase and quinone reductase) in the liver and colon. These findings suggest that the inhibitory effects of auraptene on AOM-induced colon tumorigenesis at the initiation level might be associated, in part, with increased activity of Phase II enzymes, and those at the postinitiation stage might be related to suppression of cell proliferation and lipid peroxidation in the colonic mucosa.

INTRODUCTION

Colon cancer is the third most malignant neoplasm in the world (1). It is well known that colorectal cancer is linked to a Western lifestyle, which often includes a diet high in fat (2). In Japan, the incidence of this malignancy, being the third leading cause of cancer death, has been increasing, possibly due to the Westernization of dietary habits, with a rising fat intake. On the other hand, epidemiological studies indicate an inverse correlation between the intake of fruits/vegetables and human colon cancer (3). Thus, it is considered that primary prevention including chemoprevention using the active compounds in fruits and vegetables is important for reducing this malignancy. Our search for cancer chemopreventive compounds in vegetables and fruits revealed that several compounds, including certain flavonoids, exert inhibitory effects on AOM[3]-induced colon carcinogenesis (2, 4–6). Recent findings in a short-term experiment using as an intermediate biomarker ACF, which are preneoplastic lesions for colorectal carcinoma (7–10), suggest possible chemopreventive activity of a known coumarin auraptene (Fig. 1), which is present in certain oranges: 0.04% in *Citrus natsudaidai* HAYATA, 0.01–0.02% in grapefruit, and 180 μg/100 ml in grapefruit juice (11). Citrus fruits contain other chemopreventive compounds against cancer. These include D-limonene (see Ref. 12 for review) and hesperidin (13–15). Both compounds could reduce AOM-induced ACF and/or colon carcinoma (6, 14, 16). Although antiplatelet action of auraptene has been reported (17), other biological activities, including modulatory effects on tumorigenesis in digestive organs, are not known. Recently, antipromoting effects of auraptene on 7,12-dimethylbenz[*a*]anthracene-induced mouse skin tumorigenesis has been found (11). In that study, auraptene markedly suppressed superoxide (O_2^-) generation induced by 12-*O*-tetradecanoylphorbol-13-acetate in differentiated human promyelocytic HL-60 cells (11). More recent study in our laboratory revealed an excellent antitumor activity of auraptene in 4-nitroquinoline 1-oxide-induced rat tongue carcinogenesis (18).

Free radicals, including oxyradicals, may contribute to the development of certain types of cancer (19, 20). They induce DNA sequence changes in the form of point mutations, deletions, gene amplification, and rearrangements that may result in the activation of several proto-oncogenes and/or the inactivation of some tumor suppressor genes (19). Recently, an increased mucosal oxygen free radical activity was reported in AOM-treated colonic mucosa and carcinoma tissue, and the activity was reduced by the treatment of superoxide dismutase (21).

In the present study, chemopreventive ability of auraptene on large bowel tumorigenesis was investigated in a long-term *in vivo* assay

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[3] The abbreviations used are: AOM, azoxymethane; ACF, aberrant crypt foci; ODC, ornithine decarboxylase; GST, glutathione *S*-transferase; QR, quinone reductase, which is officially designated NAD(P)H:(quinone acceptor) oxidoreductase, and also known as DT-diaphorase or menadione reductase; 4-HNE, 4-hydroxy-2(*E*)-nonenal; MDA, malondialdehyde; CDNB, 1-chloro-2,4-dinitrobenzene; DCNB, 1,2-dichloro-4-nitrobenzene.

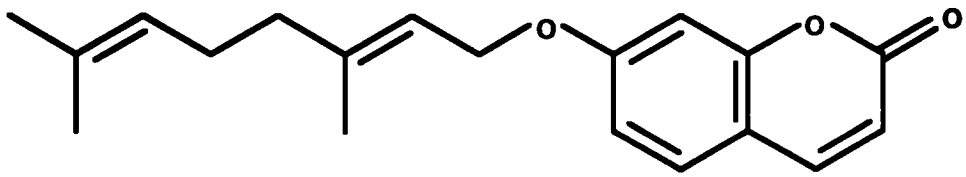

Fig. 1. Chemical structure of auraptene.

using a rat colon carcinogenesis model with an excellent colorectal carcinogen AOM, to confirm the results in our previous work (22). Also, to gain an insight into its mechanisms, we assayed the expression of cell proliferation biomarkers, such as colonic mucosal ODC activity and polyamine levels, in the colonic mucosa and blood; the activities of Phase II detoxification enzymes GST and QR, in the colonic epithelium and liver tissue; and the levels of aldehydic lipid peroxidation products, MDA and 4-HNE, in the colonic mucosa.

MATERIALS AND METHODS

Animals, Diet, and Chemicals. A total of 153 male F344 rats, 4 weeks old, obtained from Japan SLC, Inc. (Hamamatsu City, Japan), were used. Animals were housed three or four to a wire cage in an experimental room under controlled conditions of 23 ± 2°C (SD), 50 ± 10% humidity, and a 12-h light/dark cycle. They were allowed *ad libitum* access to diet and water. Powdered CE-2 (CLEA Japan, Inc., Tokyo, Japan) was used as a basal diet. AOM was purchased from Sigma Chemical Co. (St. Louis, MO). To isolate auraptene, *natsumikans* in the Wakayama Prefecture were harvested in 1994, and whole parts (4.8 kg fresh weight) were processed by using the FMC Citrus Juice Extractor (FMC Bioproducts, Rockland, ME) to give the cold-pressed oils (9.5 g). The oils thus obtained were fractionated by silica gel (*n*-hexane/ethyl acetate; stepwise method) and high-performance column chromatographies [column, μBondasphere C18 (Milford, MA), 19 × 150 mm; elute, 90% methanol in water; flow rate, 7.0 ml/min; detection, $UV_{254\ nm}$; retention time, 12.2 min; Refs. 11 and 23]. Auraptene (240 mg) was recrystallized from ethanol and identified by several spectroscopic analyses (UV, infrared, proton and carbon nuclear magnetic resonance, and mass spectrometry). The spectral data were identical with those reported previously (24). Experimental diets mixed with auraptene at two concentrations (100 and 500 ppm) were prepared by using a V-blender on a weekly basis and stored in a cold room (<4°C) until used. The diets were analyzed at the end of each week to confirm the concentration of the added auraptene, and the auraptene level was found to be quite stable (data not shown).

Experimental Procedure. After quarantine for 1 week, 128 rats age 5 weeks were divided into seven groups, as shown in Fig. 2 and in Tables 1–9. Starting at 6 weeks of age, animals in groups 1–5 were s.c. injected with AOM (15 mg/kg body weight) once a week for 3 weeks. Rats in groups 2 and 3 were given the powdered basal diet containing 100 ppm or 500 ppm auraptene for 4 weeks, beginning at 5 weeks of age. Groups 4 and 5 were fed the diets containing 100 ppm and 500 ppm auraptene for 34 weeks, respectively, starting 1 week after the last dosing of AOM. Group 6 was fed the diet containing 500 ppm auraptene alone throughout the study and did not receive AOM. Group 7 served as an untreated control. Animals were carefully observed daily and weighed weekly until they reached 14 weeks of age, and then they were weighed every 4 weeks. The consumption of experimental diets was also recorded. At the termination of the study (week 38), all of the rats were killed by decapitation. At autopsy, three or five rats from each group were randomly selected for measurement of the colonic mucosal ODC activity and the levels of MDA and 4-HNE in the colonic mucosa and for measurement of the polyamine levels in the colonic mucosa and blood. The colons, except those used for the biochemical assay, were used for scoring of ACF. After the macroscopic observation, the colonic tumors were resected for histological examination, and the remaining colonic tissues were used for biochemical assays. The intestines of the remaining rats and macroscopic lesions in other organs of all animals were embedded in paraffin, sliced 3 μm in thickness, stained with H&E, and examined histopathologically.

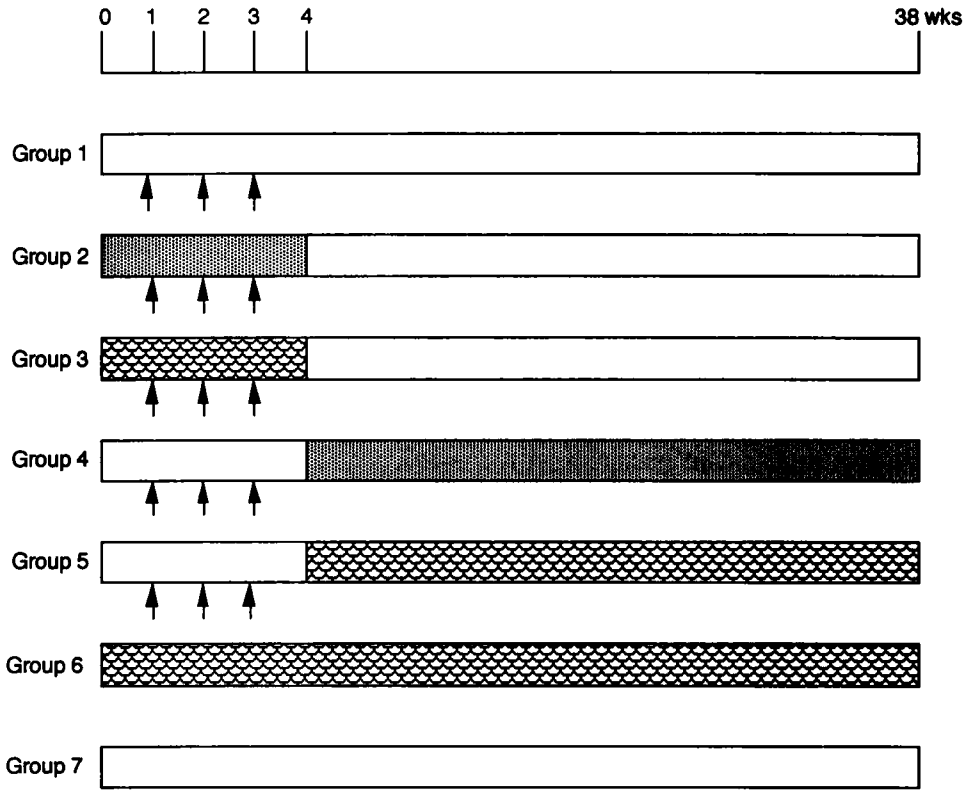

Fig. 2. Experimental protocol. ↑, AOM, 15 mg/kg body weight, s.c. injection; ▨, 100 ppm auraptene in diet; ▩, 500 ppm auraptene in diet; □, Basal diet.

ACF Analysis. At autopsy, the colons were flushed with saline, excised, cut open longitudinally along the main axis, and then washed with saline. The colons were placed between filter papers to reduce mucosal folding and were fixed in 10% buffered formalin for at least 24 h. Fixed colon sections were dipped in a 0.2% solution of methylene blue in distilled water for 1 min, briefly washed with distilled water, and placed on a microscope slide with the mucosal surface up. Using a light microscope at a magnification of ×40, ACF were distinguished by their increased size, their more prominent epithelial cells, and their increased pericryptal space compared with surrounding normal crypts (7). Crypts overlying lymphoid follicles were excluded from scoring, because normal crypts in this area can sometimes be confused with ACF. The number of ACF observed per colon, the number of aberrant crypts observed in each focus, and the location of each focus were recorded. Colons from all of the groups were scored blindly for ACF by the same observer (T. T.). After scoring, colons were processed for histopathological examination.

ODC Activity. For measurement of ODC activity, the colons were rapidly removed, slit open longitudinally, freed of all of the contents, and then rinsed in ice-cold saline. They were laid flat on a glass plate with the mucosal side up, and the mucosa was scraped with a stainless steel disposable microtome-bladed knife (S35; Feather Safety Razor Co., Ltd., Osaka, Japan). Colorectal mucosa from each rat was pooled and homogenized in 1.5 ml of 50 mM sodium phosphate buffer containing 5 mM DTT, 0.1 mM EDTA, and 0.1 mM pyridoxal 5′-phosphate using a Polytron homogenizer. The homogenates were centrifuged for 1 h at 100,000 × *g* at 4°C. The resulting cytosol fraction was used for determination of ODC activity and protein. ODC activity in the colonic mucosa was determined by the methods described previously (14). The incubation mixture in a final volume of 250 μl [50 mM sodium phosphate, 2 mM pyridoxal phosphate, 5 mM DTT, 20 mM L-ornithine, and 0.25 mCi of DL-[1-^{14}C]ornithine (specific activity, 58 mCi/mmol; Amersham Corp.)] was incubated for 1 h at 37°C. The reaction was stopped by adding 300 μl of 2 N sulfuric acid, and the $^{14}CO_2$ released was collected on barium hydroxide-saturated discs for another 30 min. ^{14}C in the form of $Ba^{14}CO_3$ was counted in a scintillation counter. The results were expressed as pmol of $^{14}CO_2$/h/mg protein.

Polyamine Levels. Polyamine levels in the colonic mucosa were assayed by the method of Koide (25). For measurement of blood polyamine level, blood from all of the rats was collected by heart puncture at sacrifice, and polyamine levels were assessed (26).

GST and QR Assay. To determine whether auraptene could modify activities of GST and QR in the liver and colon, 25 male F344 rats age 6 weeks were divided into four groups (6 or 7 rats/group), and they were gavaged with auraptene at a dose level of 0, 200, 400, or 800 mg/kg body weight in 0.5 ml of 5% gum arabic (Sigma Chemical Co.) for 5 consecutive days. All rats were killed by decapitation 30 min after the last gavage. At sacrifice, the livers and colons were excised immediately. The liver was perfused with saline to remove blood and minced into small pieces. The colon was slit longitudinally and washed with PBS (pH 7.4), and mucosa was collected by scraping the mucosal surface using a stainless steel disposable microtome bladed knife (S35; Feather Safety Razor Co.). Aliquots of minced liver and mucosal scrapings were processed for cytosolic fraction as described (27, 28). The activities of GST with CDNB and/or DCNB as substrates and QR with NADH and menadione as substrates were determined as described (29–31). All assays were performed with spectrophotometry at 340 nm, and all samples were measured in triplicate. One unit of enzyme activity is the amount of enzyme catalyzing the conversion of 1 μmol substrate to product per min at 25°C. Cytosolic protein concentrations were determined by the Bradford method (32) using BSA as the standard.

Determination of Aldehydic Lipid Peroxidation Products. The levels of the sum of MDA and 4-HNE in the colonic mucosa were determined by using a BIOTECH LPO-586 experimental kit (OXIS International, Inc., Portland, OR; Ref. 33). Briefly, each tissue sample was washed with ice-cold 0.9% NaCl and minced in 1 ml of ice-cold 20 mM Tris-HCl buffer (pH 7.4). Minced tissue was homogenized and centrifuged at 3000 × *g* at 4°C for 10 min. Two hundred μl of supernatant were added to 650 μl of 10 mM *N*-methyl-2-phenylindole and then to 150 μl of 15.4 M methanesulfonic acid. The mixture thus obtained was incubated at 45°C for 40 min. After cooling on ice, the sample was centrifuged at 3000 × *g* at 4°C for 10 min, and the clear supernatants were used for the measurement of absorbance at 586 nm. The protein content in supernatant was measured by using the Bio-Rad protein assay (Bio-Rad Laboratories, Hercules, CA).

Statistical Methods. Where applicable, data were analyzed using Fisher's exact probability test, Student's *t* test, or Welch's *t* test, taking $P < 0.05$ as the level of significance.

RESULTS

General Observations. During the study, clinical signs of toxicity, low survival, and poor condition were not observed in any groups. This was confirmed by histopathological examinations in liver, kidneys, heart, and lungs of the rats. The mean daily intake of a diet with or without auraptene per rat was between 15.1 and 15.6 g/day/rat (mean intake of auraptene/rat/day: 100-ppm diet, 1.534 mg; 500-ppm diet, 7.673 mg). Mean body and liver weights in all groups at sacrifice are shown in Table 1. There were no significant differences on the mean body weights among the groups. The mean liver weight of group 1 (AOM alone) was significantly smaller than that of group 7 (untreated; $P < 0.001$). The mean liver weight in group 3 (AOM + 500 ppm auraptene) was significantly increased when compared with that of group 1 ($P < 0.001$). The mean percentage liver weights (g/100 g body weight) of groups 3, 4 (AOM → 100 ppm auraptene), and 5 (AOM → 500 ppm auraptene) were significantly greater than that of group 1 ($P < 0.001$, $P < 0.01$, and $P < 0.005$, respectively). This value in group 1 was significantly smaller than that of group 7 ($P < 0.001$).

Incidence and Multiplicity of Intestinal Neoplasms. Macroscopic observation revealed that most tumors developed in the large intestine (mainly in the middle and distal colon) and some in the small intestine of rats in groups 1–5. Animals in groups 6 and 7 did not have neoplasms in any organs examined. Colon tumors were sessile or pedunculated tumors and histologically tubular adenoma, adenocarcinoma, or signet ring-cell carcinoma, with a higher incidence of adenocarcinoma. A few rats had renal mesenchymal tumors and/or preneoplastic hepatocellular lesions in groups 1–5, but these were not found in groups 6 and 7. The incidence and multiplicity of intestinal tumors are shown in Tables 2 and 3. The frequencies of large intestinal adenocarcinoma in groups 3 (20%), 4 (24%), and 5 (20%) were significantly smaller than that in group 1 (59%; $P = 0.0075$, $P = 0.021$, and $P = 0.0075$, respectively). The frequency of colorectal adenocarcinoma in group 2 (35%) was lower than in group 1, but a significant difference was not present ($P = 0.099$). The incidence of small intestinal adenocarcinoma in group 3 (5%) was significantly lower than in group 1 (37%; $P = 0.010$), but those in groups 2 (15%), 4 (12%), and 5 (15%) did not significantly differ from that in group 1. As presented in Table 3, significant reduction in the multiplicities of colorectal carcinoma (number of carcinomas/rat) in groups 3 (0.20 ± 0.40), 4 (0.24 ± 0.42), and 5 (0.20 ± 0.40) and that of small intestinal adenocarcinoma in group 3 (0.05 ± 0.22) was found when compared with group 1 (colorectal carcinoma, 0.56 ± 0.48; small intestinal carcinoma, 0.30 ± 0.46; $P < 0.01$, $P < 0.05$, $P < 0.01$, and $P < 0.02$, respectively).

Frequency of ACF at the End of the Study. The incidence of ACF at the end of the study is listed in Table 4. ACF developed in rats treated with AOM together with, or without, auraptene (groups 1–5), whereas no ACF were present in rats treated without AOM (groups 6 and 7). The frequencies of ACF/colon, the number of ACF/cm^2, the number of aberrant crypts/colon, and the number of aberrant crypts/focus in groups 2–5 were significantly smaller than those of group 1 ($P < 0.01$ or $P < 0.001$).

Colonic Mucosal ODC Activity. As indicated in Table 5, AOM treatment significantly increased the colonic mucosal ODC activity ($P < 0.001$). Dietary feeding of auraptene during both the initiation

Table 1 *Body, liver, and relative liver weights of rats treated with AOM and/or auraptene*[a]

Group	Treatment	No. of rats examined	Body weight (g)	Liver weight (g)	Relative liver weight (g/100 g body weight)
1	AOM alone	27	334 ± 17	11.4 ± 1.5[b]	3.32 ± 0.32[b]
2	AOM + 100 ppm auraptene	20	341 ± 18	11.9 ± 1.6	3.49 ± 0.38
3	AOM + 500 ppm auraptene	20	358 ± 20	13.6 ± 1.7[c]	3.79 ± 0.36[c]
4	AOM→100 ppm auraptene	17	328 ± 25	12.1 ± 1.5	3.69 ± 0.44[d]
5	AOM→500 ppm auraptene	20	326 ± 39	11.7 ± 1.3	3.59 ± 0.28[e]
6	500 ppm auraptene	12	346 ± 25	13.3 ± 2.0	3.85 ± 0.55
7	No treatment	12	336 ± 22	13.0 ± 0.8	3.87 ± 0.23

[a] Mean ± SD.
[b] Significantly different from group 7 by Student's t test or Welch's t test ($P < 0.001$).
[c] Significantly different from group 1 by Student's t test ($P < 0.001$).
[d] Significantly different from group 1 by Student's t test ($P < 0.01$).
[e] Significantly different from group 1 by Student's t test ($P < 0.005$).

Table 2 *Incidence of intestinal neoplasms of rats fed auraptene during or after AOM exposure*[a]

			No. of rats with intestinal neoplasms at:								
			Entire intestine			Small intestine			Large intestine		
Group	Treatment	No. of rats	Total	AD	ADC	Total	AD	ADC	Total	AD	ADC
1	AOM alone	27	18 (67%)	1 (4%)	18 (76%)	8 (30%)	0	10 (37%)	16 (59%)	1 (4%)	16 (59%)
2	AOM + 100 ppm auraptene	20	11 (55%)	3 (15%)	9 (39%)	3 (15%)	0	3 (15%)	9 (45%)	3 (15%)	7 (35%)
3	AOM + 500 ppm auraptene	20	7 (35%)[b]	2 (10%)	5 (47%)[c]	1 (5%)[d]	0	1 (5%)[e]	6 (30%)[b]	2 (10%)	4 (20%)[f]
4	AOM→100 ppm auraptene	17	7 (41%)	1 (6%)	6 (56%)[b]	2 (12%)	0	2 (12%)	5 (29%)	1 (6%)	4 (24%)[g]
5	AOM→500 ppm auraptene	20	10 (50%)	3 (15%)	7 (15%)[b]	4 (20%)	1 (5%)	3 (15%)	6 (30%)[b]	2 (10%)	4 (20%)[f]
6	500 ppm auraptene	12	0	0	0	0	0	0	0	0	0
7	No treatment	12	0	0	0	0	0	0	0	0	0

[a] AD, adenoma; ADC, adenocarcinoma.
[b] Significantly different from group 1 by χ^2 test ($P < 0.05$).
[c] Significantly different from group 1 by Fisher's exact probability test ($P = 0.005$).
[d] Significantly different from group 1 by Fisher's exact probability test ($P = 0.036$).
[e] Significantly different from group 1 by Fisher's exact probability test ($P = 0.010$).
[f] Significantly different from group 1 by Fisher's exact probability test ($P = 0.0075$).
[g] Significantly different from group 1 by Fisher's exact probability test ($P = 0.021$).

(groups 2 and 3) and postinitiation phases (groups 4 and 5) significantly decreased this increase ($P < 0.002$ or $P < 0.001$). The ODC activity of rats in group 6 (auraptene alone) was slightly greater than group 7 (untreated control).

Polyamine Levels in the Colonic Mucosa and Blood. As summarized in Table 6, the total polyamine levels of colonic mucosa and blood in group 1 were significantly greater than that of untreated control (group 7; $P < 0.001$ and $P < 0.002$, respectively). The mean total polyamine concentrations of colonic mucosa in groups 2–5 were significantly lower than that of group 1 ($P < 0.01$, $P < 0.05$, or $P < 0.001$). Total blood polyamine levels in groups 2–5 were also significantly smaller than that of group 1 ($P < 0.01$, $P < 0.05$, or $P < 0.005$). Spermidine levels of colonic mucosa and blood in group 1 and mucosal spermidine level in group 6 were significantly increased when compared to that of group 7 ($P < 0.001$, $P < 0.005$, or $P < 0.05$). Auraptene feeding during the postinitiation phase significantly decreased this increase in colonic mucosa ($P < 0.005$ or $P < 0.001$). Blood spermidine levels of groups 2, 3, and 5 were significantly lower than that of group 1 ($P < 0.05$ or $P < 0.01$). Mucosal spermine levels in groups 1 and 6 were significantly higher than in group 7 ($P < 0.001$ and $P < 0.005$, respectively). The average values of total polyamine and diamine in groups 6 and 7 were almost comparable.

GST and QR Activities. GST and QR activities in the liver and colon of rats gavaged with various dose levels of auraptene are listed in Tables 7 and 8. Dosing of 400 and 800 mg/kg body weight of auraptene significantly increased liver GST (GST-CDNB and GST-DCNB) and QR activities ($P < 0.002$, $P < 0.001$, or $P < 0.005$). Similarly, gavaged auraptene significantly elevated the GST-CDNB and QR activities in the colonic mucosa ($P < 0.005$, $P < 0.02$, or $P < 0.05$).

Table 3 *Multiplicity of intestinal neoplasms of rats fed auraptene during or after AOM exposure*[a]

		Multiplicity (No. of neoplasms/rat) of neoplasms at:								
		Entire intestine			Small intestine			Large intestine		
Group	Treatment (No. of rats examined)	Total	AD	ADC	Total	AD	ADC	Total	AD	ADC
1	AOM alone (n = 27)	0.91 ± 0.81	0.06 ± 0.24	0.85 ± 0.74	0.30 ± 0.46	0	0.30 ± 0.46	0.62 ± 0.59	0.06 ± 0.24	0.56 ± 0.48
2	AOM + 100 ppm auraptene (n = 20)	0.75 ± 0.76	0.15 ± 0.36	0.60 ± 0.73	0.20 ± 0.51	0	0.20 ± 0.51	0.60 ± 0.73	0.20 ± 0.51	0.40 ± 0.58
3	AOM + 500 ppm auraptene (n = 20)	0.35 ± 0.48[b]	0.10 ± 0.30	0.25 ± 0.43[c]	0.05 ± 0.22[c]	0	0.05 ± 0.22[c]	0.30 ± 0.46	0.10 ± 0.30	0.20 ± 0.40[d]
4	AOM→100 ppm auraptene (n = 17)	0.41 ± 0.49[c]	0.06 ± 0.24	0.35 ± 0.48[b]	0.12 ± 0.32	0	0.12 ± 0.32	0.29 ± 0.46	0.06 ± 0.24	0.24 ± 0.42[b]
5	AOM→500 ppm auraptene (n = 20)	0.50 ± 0.50[e]	0.15 ± 0.36	0.35 ± 0.48[f]	0.20 ± 0.40	0.05 ± 0.22	0.15 ± 0.36	0.30 ± 0.46	0.10 ± 0.51	0.20 ± 0.40[d]
6	500 ppm auraptene (n = 12)	0	0	0	0	0	0	0	0	0
7	No treatment (n = 12)	0	0	0	0	0	0	0	0	0

[a] AD, adenoma; ADC, adenocarcinoma. Values are means ± SD.
[b] Significantly different from group 1 by Students t test ($P < 0.05$).
[c] Significantly different from group 1 by Welch's t test ($P < 0.02$).
[d] Significantly different from group 1 by Student's t test ($P < 0.01$).
[e] Significantly different from group 1 by Welch's t test ($P < 0.05$).
[f] Significantly different from group 1 by Welch's t test ($P < 0.01$).

Table 4 *Effect of auraptene on the development of AOM-induced ACF at the end of study*[a]

Group	Treatment	Incidence	No. of ACF/colon	No. of ACF/cm^2	No. of aberrant crypts/colon	No. of aberrant crypts/focus
1	AOM alone	20 of 20 (100%)	90 ± 15	6.53 ± 1.76	265 ± 73	2.94 ± 0.21
2	AOM + 100 ppm auraptene	15 of 15 (100%)	66 ± 10[b]	5.24 ± 0.93[c]	150 ± 33[b]	2.27 ± 0.19[b]
3	AOM + 500 ppm auraptene	15 of 15 (100%)	57 ± 6[b]	4.78 ± 0.35[b]	124 ± 19[b]	2.18 ± 0.10[b]
4	AOM→100 ppm auraptene	12 of 12 (100%)	53 ± 9[b]	4.63 ± 0.81[b]	116 ± 14[b]	2.19 ± 0.13[b]
5	AOM→500 ppm auraptene	15 of 15 (100%)	50 ± 10[b]	4.60 ± 0.42[b]	103 ± 18[b]	2.06 ± 0.23[b]
6	500 ppm auraptene	0 of 7 (0%)	0	0	0	0
7	No treatment	0 of 7 (0%)	0	0	0	0

[a] Mean ± SD.
[b] Significantly different from group 1 by Student's *t*-test ($P < 0.001$).
[c] Significantly different from group 1 by Welch's *t* test ($P < 0.01$).

Table 5 *Colonic mucosal ODC activities in rats treated with AOM and/or auraptene*

Group	Treatment	No. of rats examined	ODC activity (pmol $^{14}CO_2$/h/mg protein)[a]
1	AOM alone	5	62.2 ± 10.0[b]
2	AOM + 100 ppm auraptene	5	32.3 ± 3.9[c]
3	AOM + 500 ppm auraptene	5	24.1 ± 4.1[d]
4	AOM→100 ppm auraptene	5	21.2 ± 4.1[d]
5	AOM→500 ppm auraptene	5	19.2 ± 5.0[d]
6	500 ppm auraptene	5	7.5 ± 3.4
7	No treatment	5	5.2 ± 1.8

[a] Mean ± SD.
[b] Significantly different from group 7 by Welch's *t* test ($P < 0.001$).
[c] Significantly different from group 1 by Student's *t* test ($P < 0.002$).
[d] Significantly different from group 1 by Welch's *t* test ($P < 0.001$).

Levels of MDA and 4-HNE. The levels of the sum of MDA and 4-HNE are shown in Table 9. In the colonic mucosa of rats treated with AOM alone (group 1), a significant increase in the sum of MDA and 4-HNE was observed ($P < 0.01$). Auraptene feeding significantly lowered the amount of these products ($P < 0.005$, $P < 0.01$, or $P < 0.02$).

DISCUSSION

The results in the present study clearly indicate that dietary feeding of auraptene effectively suppressed the occurrence of ACF and colorectal carcinomas induced by AOM when administered during or after the carcinogen treatment. The inhibition was dose dependent. The results confirm our earlier report (22) showing that auraptene exerts a strong inhibitory effect on colonic ACF, which are the possible precursor lesions for colorectal adenocarcinoma in rodents and humans (7) and a reliable biological marker for screening chemopreventive agents against colorectal adenocarcinoma (5, 9, 34, 35).

As pointed out by Wattenberg (36) and Talalay (37), several mechanisms by which chemopreventive agents exert their inhibitory effects on tumorigenesis could be considered. The metabolic activation of AOM to a reactive species capable of alkylating DNA occurs through the hydroxylation of AOM to methylazoxymethanol in the liver, and the metabolism of methylazoxymethanol to a highly reactive electrophile, *i.e.*, methyldiazonium iron (an ultimate carcinogen that can methylate cellular nucleophiles, including DNA) occurs in the liver and colon (38, 39). Several colon tumor inhibitors, including disulfiram, have been reported to alter AOM metabolism and inhibit tumorigenicity (5, 40–42). Certain organosulfur compounds, including oltipraz, also exert their chemopreventive potential by this mechanism (43). As observed in the present study, administration of auraptene significantly increased dose dependently the activities of detoxification enzymes QR and GST in the liver and colon. Thus, the inhibitory effect of auraptene feeding during the AOM treatment might be due to the blocking of some initiation events by enhancement of QR and GST activities in the liver and colon. In fact, auraptene could reduce the frequency of micronuclei induced by AOM exposure in our previous study (22). Other chemopreventive agents, *e.g.*, organosulfur compounds containing allyl groups, could inhibit colon carcinogenesis through induction of GST activity in both the liver and colon (44). Interestingly, Szarka *et al.* (45) reported that the GST activity in blood lymphocytes is reduced in humans at increased risk for colon cancer and correlated with their colonic mucosal GST activity.

Auraptene is reported to have antiplatelet action (17) and a strong antioxidative effect (11). Certain cancer chemopreventers with antioxidative activity could inhibit carcinogenesis by altering the increased cell proliferation activity in their target organs (6). In the present study, expression of cell proliferation biomarkers such as ODC activity and polyamine level in the colonic mucosal epithelium was significantly inhibited by the dietary feeding of this chemical. Also, the level of total polyamine in the colonic mucosa was well correlated with that in blood, suggesting that blood polyamine level may be one of the intermediate biomarkers in chemopreventive studies (6).

Oxidative stress and lipid peroxidation have been suggested to play a

Table 6 *Tissue and blood polyamine levels*

Group	Treatment	No. of rats examined	Polyamine levels Tissue (nmol/mg protein)/blood (nmol/ml)[a] Total	Diamine	Spermidine	Spermine
1	AOM alone	7	30.8 ± 2.0[b]/38.6 ± 9.1[c]	0.4 ± 0.1/0.8 ± 1.5	21.3 ± 2.4[b]/29.0 ± 6.8[d]	9.1 ± 0.3[b]/8.8 ± 5.9
2	AOM + 100 ppm auraptene	5	26.9 ± 2.0[e]/26.2 ± 1.9[e]	0.1 ± 0.1/0.1 ± 0.3	19.6 ± 2.4/22.0 ± 1.8[f]	7.2 ± 1.5[f]/4.0 ± 0.7
3	AOM + 500 ppm auraptene	5	28.0 ± 1.4[f]/23.8 ± 3.8[e]	0.2 ± 0.3/0.1 ± 0.2	21.2 ± 0.9/20.1 ± 2.9[f]	6.5 ± 1.1[e]/3.6 ± 1.5
4	AOM→100 ppm auraptene	5	21.5 ± 7.5[e]/28.0 ± 3.5[f]	1.5 ± 1.7/0.0 ± 0.0	13.2 ± 3.8[g]/23.4 ± 3.0	6.9 ± 3.3/4.6 ± 1.2
5	AOM→500 ppm auraptene	5	15.5 ± 2.5[h]/21.7 ± 2.6[g]	1.8 ± 1.9/0.0 ± 0.0	9.3 ± 5.6[h]/18.3 ± 1.8[e]	9.5 ± 0.6/3.5 ± 0.8
6	500 ppm auraptene	5	13.5 ± 2.6/19.1 ± 1.9	0.1 ± 0.2/0.0 ± 0.0	7.0 ± 1.3[i]/16.3 ± 2.3	6.4 ± 1.5[d]/2.8 ± 1.0
7	No treatment	5	13.2 ± 1.4/20.4 ± 1.7	0.1 ± 0.1/0.0 ± 0.0	10.3 ± 2.2/16.2 ± 2.8	2.9 ± 1.2/4.2 ± 1.6

[a] Mean ± SD.
[b] Significantly different from group 7 by Student's *t* test or Welch's *t* test ($P < 0.001$).
[c] Significantly different from group 7 by Student's *t* test or Welch's *t* test ($P < 0.002$).
[d] Significantly different from group 7 by Student's *t* test or Welch's *t* test ($P < 0.005$).
[e] Significantly different from group 1 by Student's *t* test or Welch's *t* test ($P < 0.01$).
[f] Significantly different from group 1 by Student's *t* test or Welch's *t* test ($P < 0.05$).
[g] Significantly different from group 1 by Student's *t* test or Welch's *t* test ($P < 0.005$).
[h] Significantly different from group 1 by Student's *t* test or Welch's *t* test ($P < 0.001$).
[i] Significantly different from group 7 by Student's *t* test or Welch's *t* test ($P < 0.05$).

Table 7 *GST and QR activities in liver of rats gavaged with auraptene*

Enzymes	Dose levels[a] 0 mg/kg bw	200 mg/kg bw	400 mg/kg bw	800 mg/kg bw
GST-CDNB	817.3 ± 124.9 (6)	921.8 ± 145.5 (6)	1266.1 ± 208.2[b,c] (6)	1332.2 ± 234.6[d,e] (7)
GST-DCNB	39.1 ± 2.9 (6)	39.4 ± 7.7 (6)	57.2 ± 6.1[c,f] (6)	57.2 ± 8.7[e,g] (7)
QR	127.0 ± 12.4 (6)	213.7 ± 47.2[g] (6)	239.1 ± 22.8[d] (6)	298.3 ± 40.4[c,d,h] (7)

[a] Dose levels of auraptene [mg/kg body weight (bw)/day] for 5 days orally; rats were killed 30 min after the last administration. Numbers in parentheses are numbers of rats examined.
[b] Significantly different from the 0 mg group by Student's *t* test or Welch's *t* test ($P < 0.002$).
[c] Significantly different from the 200 mg group by Student's *t* test ($P < 0.01$).
[d] Significantly different from the 0 mg group by Student's *t* test or Welch's *t* test ($P < 0.001$).
[e] Significantly different from the 200 mg group by Student's *t* test ($P < 0.005$).
[f] Significantly different from the 200 mg group by Student's *t* test ($P < 0.002$).
[g] Significantly different from the 0 mg group by Student's *t* test or Welch's *t* test ($P < 0.005$).
[h] Significantly different from the 400 mg group by Student's *t* test ($P < 0.01$).

role in carcinogenesis. Free radical lipid peroxidation might be involved in tumor promotion and progression of carcinogenesis (19, 20, 46). 4-HNE is produced as a major product of the peroxidative decomposition of ω6 polyunsaturated fatty acids and possesses cytotoxic, hepatotoxic, mutagenic, and genotoxic properties. Increased levels of 4-HNE were found in plasma and various organs under conditions of oxidative stress. MDA is in many instances the most abundant individual aldehyde resulting from lipid peroxidation. In *in vitro* studies, MDA can alter proteins, DNA, RNA, and many other biomolecules. Increased levels of the products of lipid peroxidation, including 4-HNE and MDA, were found in colon (47, 48), liver (48, 49), breast (50), and kidney (51) carcinogenesis. In fact, MDA and prostaglandin E_2 levels were significantly higher than in human colorectal cancer tissue when compared to normal colonic mucosa (52). In the present study, AOM treatment significantly elevated the amount of the aldehydic lipid peroxidation products MDA and 4-HNE, suggesting that AOM causes oxidative stress in the whole colonic mucosa (21). In the current study, auraptene feeding significantly reduced the increased levels of these aldehydic lipid peroxidation products. Thus, the cancer inhibitory effect of auraptene during the postinitiation stage may be due to the modification of hyper-cell proliferation in the colorectal mucosa exposed to AOM through suppression of oxidative stress. Recently, green tea extract was reported to inhibit colonic mucosal lipid hyperoxidation during 1,2-dimethylhydrazine-induced rat colon carcinogenesis (53). Canthaxanthin, a chemopreventive compound for colon carcinogenesis (54), is also protective against peroxidation (55).

Eicosanoids have been implicated in colon carcinogenesis. Arachidonic acid, a precursor of several biologically active eicosanoids, is the most abundant polyenoic fatty acid found in the phospholipids of mammalian tissues. It is metabolized both by the cyclooxygenase pathway producing prostaglandins, thromboxane, and prostacyclin and by the lipoxygenase pathway leading to the formation of leukotrienes and lipoxins. Arachidonic acid products synthesized via both pathways could modulate the colon carcinogenesis (56, 57), and some inhibitors, including cyclooxygenase 2 inhibitors (58), of the arachidonic acid cascade (59, 60) possess chemopreventive activity in colon carcinogenesis. In this context, the effects of auraptene on arachidonate metabolism, particularly via cyclooxygenase and/or lipoxygenase pathways, are of interest and are important for considering possible mechanisms of inhibitory effect of auraptene found in the present study.

Table 8 *GST and QR activities in colon of rats gavaged with auraptene*

Enzymes	Dose levels[a] 0 mg/kg bw	200 mg/kg bw	400 mg/kg bw	800 mg/kg bw
GST-CDNB	112.9 ± 16.2 (6)	126.2 ± 7.9 (6)	147.4 ± 12.7[b,c] (6)	149.6 ± 19.9[b,d] (7)
QR	548 ± 69 (6)	570 ± 53 (6)	680 ± 82[e,d] (6)	707 ± 144[f] (7)

[a] Dose levels of auraptene [mg/kg body weight (bw)/day] for 5 days orally; rats were killed 30 min after the last administration. Numbers in parentheses are numbers of rats examined.
[b] Significantly different from the 0 mg group by Student's *t* test ($P < 0.005$).
[c] Significantly different from the 200 mg group by Student's *t* test ($P < 0.002$).
[d] Significantly different from the 200 mg group by Student's *t* test ($P < 0.05$).
[e] Significantly different from the 0 mg group by Student's *t* test ($P < 0.02$).
[f] Significantly different from the 0 mg group by Student's *t* test ($P < 0.05$).

Table 9 *Effect of auraptene on the amounts of MDA and 4-HNE in the colonic mucosa of rats treated with AOM*

Group	Treatment	No. of rats examined	MDA + 4-HNE (nmol/mg protein)[a]	Relative value
1	AOM alone	5	21.3 ± 8.1[b]	760
2	AOM + 100 ppm auraptene	5	3.1 ± 2.2[c]	111
3	AOM + 500 ppm auraptene	5	2.1 ± 0.8[d]	75
4	AOM→100 ppm auraptene	5	2.5 ± 1.4[d]	89
5	AOM→500 ppm auraptene	5	5.1 ± 0.9[e]	179
6	500 ppm auraptene	3	4.6 ± 2.3	164
7	No treatment	3	2.8 ± 1.1	100

[a] Mean ± SD.
[b] Significantly different from group 7 by Welch's *t* test ($P < 0.01$).
[c] Significantly different from group 1 by Welch's *t* test ($P < 0.005$).
[d] Significantly different from group 1 by Welch's *t* test ($P < 0.01$).
[e] Significantly different from group 1 by Welch's *t* test ($P < 0.02$).

In summary, dietary administration of auraptene significantly suppressed the development of AOM-induced rat colonic carcinoma in conjunction with reduced expression of cell proliferation biomarkers in the colorectal mucosa and induction of the Phase II enzymes QR and GST in the liver and large intestine, and reduced level of aldehydic lipid peroxidation products in the colorectal mucosa. Although additional studies on chronic toxicity, metabolism, and tissue distribution of auraptene are needed, the results described here provide the evidence of a novel chemopreventive agent against colorectal tumorigenesis. Our collaborative ongoing studies on the effect of auraptene on cyclooxygenase 2 expression during rat colon carcinogenesis and the possible metabolic pathways for auraptene will help to understand the chemopreventive mechanism of action for this compound.

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