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Chemoprevention of 4-nitroquinoline 1-oxide-induced oral carcinogenesis by citrus auraptene in rats

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The modifying effects of citrus auraptene given during the initiation and post-initiation phases of oral carcinogenesis initiated with 4-nitroquinoline 1-oxide (4-NQO) were investigated in male F344 rats. At 6 weeks of age, animals were divided into experimental and control groups, and fed the diets containing 100 ppm or 500 ppm auraptene. At 7 weeks of age, all animals except those treated with auraptene alone and control groups were given 4-NQO (20 ppm) in the drinking water for 8 weeks to induce tongue carcinoma. Starting 7 days before the 4-NQO exposure, groups of animals were fed the diets containing auraptene (100 and 500 ppm) for 10 weeks and then switched to the basal diet. Starting 1 week after the cessation of 4-NQO exposure, the groups given 4-NQO and a basal diet were switched to the diets mixed with auraptene (100 and 500 ppm), and maintained on these diets for 22 weeks. The other groups consisted of rats fed auraptene alone (500 ppm) or untreated rats. All rats were necropsied at the termination of the study (week 32). The incidences of tongue lesions (neoplasms and preneoplasms), polyamine levels in the tongue tissue and cell proliferation activity estimated by 5-bromodeoxyuridine (BrdU)-labelling index were compared among the groups. In addition, the activities of gluthathione *S*-transferase (GST) and quinone reductase (QR) in liver and tongue of rats gavaged various doses of auraptene (0, 200, 400 and 800 mg/kg body wt) for 5 days were assayed. Feeding of auraptene at both doses during the initiation phase caused a significant reduction in the frequency of tongue carcinoma (100 ppm auraptene, 91% reduction, $P < 0.001$; 500 ppm auraptene, 63% reduction, $P < 0.05$). When fed auraptene after 4-NQO exposure, the frequency of tongue carcinoma was also decreased (100 ppm auraptene, 100% reduction, $P < 0.001$; 500 ppm auraptene, 74% reduction, $P < 0.01$). The incidences of tongue severe dysplasia in these groups were significantly smaller than those in carcinogen controls ($P < 0.05$). There were no pathological alterations in rats treated with 500 ppm auraptene alone or those in an untreated control group. Dietary administration of auraptene significantly decreased BrdU-labelling index and polyamine concentrations in the oral mucosa ($P < 0.05$). In addition, auraptene administration significantly increased the activities of GST and QR in the liver and tongue. Although dose-dependent effect was not found, citrus auraptene is effective in inhibiting the development of oral neoplasms induced by 4-NQO. Thus, suppression by the initiation-feeding of auraptene might relate to elevation in the phase II enzymes GST and QR of the liver and tongue, and inhibition occurring during the post-initiation might be related to suppression of increased cell proliferation caused by 4-NQO in the oral mucosa.

Introduction

In contrast to certain areas, such as South-east Asia, the Pacific Islands, parts of Europe, and parts of Brazil where oral cancer is a common neoplasm (1), Japan has one of the lowest incidences of oral, lip, and pharyngeal cancers in the world (2). However, the patients with these malignancies have recently been increasing from 3.6/100,000 population in 1985 to 6.0/100,000 population in 1987 (3). Use of smokeless tobacco is reported to increase the incidence of oral cancer, especially tongue cancer among young adults (4). Neoplasms in the head and neck, including the oral cavity are known to have several biological characteristics that are multistage and multifocal carcinogenesis (5), suggesting that patients cured by primary treatment will develop a second cancer within a few years. Therefore, it is necessary to embrace new ideas that will change the prognosis of this malignancy. One such promising approach is chemoprevention (6).

In a large number of epidemiological data on the relations between diet and cancer, a protective effect of the consumption of vegetables and fruits on various forms of cancer, including oral cancer is suggested (7–9). In fact, several natural compounds present in fruits and vegetables are reported to inhibit chemical carcinogenesis (see ref. nos 10–14 for review). Recent studies in our laboratory indicate that some flavonoids in daily diets have an inhibitory effect on oral carcinogenesis (12,15–17). Thus, vegetables and fruits are rich sources for chemopreventive agents against certain cancers (13,18,19). Limonene, which is a monocyclic monoterpene found in the essential oils of *Citrus* fruits, spices and herbs, is known to be an inhibitor of chemically-induced skin, breast, forestomach, lung and liver carcinogenesis in rodents (see ref. no. 20 for review). *d*-Limonene causes 32% reduction in the frequency of aberrant

***Abbreviations:** 4-NQO, 4-nitroquinoline 1-oxide; BrdU, 5-bromo-2′-deoxyuridine; GST, glutathione *S*-transferase; QR, quinone reductase, which is officially designated NAD(P)H:(quinone acceptor) oxidoreductase (also known as DT-diaphorase or menadione reductase); CDNB, 1-chloro-2,4-dinitrobenzene; DCNB, 1,2-dichloro-4-nitrobenzene; ODC, ornithine decarboxylase.

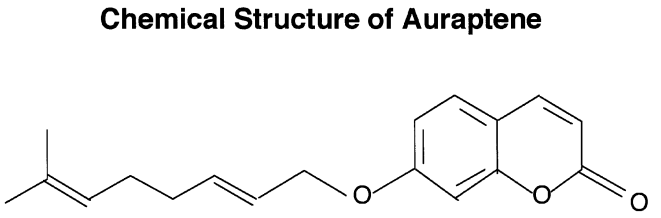

Fig. 1. Chemical structures of auraptene.

crypt foci, which are considered to be precursor lesions of colon carcinoma and is suggestive to be a possible cancer chemopreventive agent against colon cancer (21). However, limonene-induced nephrotoxicity was reported in rats harboring α_{2u}-globulin (22). Their nephrotoxicity is male rat specific and does not extrapolate to either mice or primates (22). Ninety to ninety-five per cent of limonene is found in orange peel. Hesperidin, another compound in the peel of *Citrus* fruits, also could effectively suppress aberrant crypt foci development (61% reduction) and has been found to inhibit azoxymethane-induced colon carcinogenesis (23,24). A known coumarin auraptene (Figure 1) is also present in certain orange peels, but its amount is small: 0.04% in *C.natsudaidai* HAYATA, 0.01-0.02% in grapefruit, and 180 μg/100 ml in grapefruit juice. Anti-platelet action of auraptene has been reported (25), but other biological activities, including modulatory effects on tumorigenesis in digestive organs is not known. Recently, anti-promoting effects of auraptene on 7,12-dimethylbenz[*a*]anthracene-induced mouse skin tumorigenesis has been found (26). Also, auraptene markedly suppressed superoxide generation induced by 12-*O*-tatradecanoylphorbol-13-acetate in differentiated human promyelocytic HL-60 cells (26). More recent study revealed that auraptene effectively inhibited aberrant crypt foci (27).

In a short-term pilot study, auraptene inhibited the development of tongue dysplasia induced by 4-nitroquinoline 1-oxide (4-NQO*). Therefore, in the present study, possible chemopreventive effect of auraptene on 4-NQO-induced oral (tongue) carcinogenesis was investigated in male F344 rats. The cell proliferation biomarker's expression such as 5-bromo-2′-deoxyuridine (BrdU)-labelled nuclei of the tongue squamous epithelium and polyamines level in the tongue mucosa was also examined. Since certain chemopreventive agents exert their inhibitory effects of carcinogenesis through increase of activities of phase II enzymes such as glutathione *S*-transferase (GST) and quinone reductase (QR) (28), the effect of auraptene on GST and QR activities was measured in the liver and tongue mucosa.

Materials and methods

A pilot study

Twenty F344 rats aged 6 weeks (Japan SLC, Inc., Hamamatsu City, Japan) were used to test the modifying effect of auraptene on the occurrence of tongue dysplasia induced by 4-NQO. Fifteen animals were given 4-NQO (20 ppm in drinking water), with or without auraptene (100 ppm or 500 ppm in diet), for 10 weeks. The dose levels were determined on the basis of our previous data (27). Remaining animals (five rats) were served as untreated control. All rats were killed 10 weeks after the start, and the incidence of tongue dysplasia was determined on haematoxylin and eosin-stained section.

Animals, diets and carcinogen

Male F344 rats (4 weeks old) were purchased from Japan SLC, Inc. (Hamamatsu City, Japan). After 2 weeks quarantine, they were transferred to the holding room under controlled condition at 23 ± 2°C (SD) temperature, 50 ± 10% humidity and a 12-h light/dark cycle, and housed in groups of three or four per cage. They were sorted out on wt basis into experimental and control groups, and given tap water and powdered CE-2 diet from CLEA Japan, Inc. (Tokyo, Japan). The basal diet CE-2 (50.4% crude carbohydrate, 24.8% crude protein, 4.6% crude fat, 7.2% ash, 4.2% crude cellulose and 8.8% water) did not contain auraptene. To isolate auraptene, *natsumikans* in Wakayama Prefecture in Japan were harvested in 1994 and whole parts (4.8 kg fresh wt) were processed by using the FMC Citrus Juice Extractor (FMC Co., CH) 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, Waters, MA, 19×150 mm; elute: 90% methanol in water; flow rate: 7.0 ml/min; detection: UV_{254nm}; retention time: 12.2 min). Auraptene (240 mg) was recrystallized from ethanol and identified by several spectroscopic analyses (UV, IR, proton- and carbon-nuclear magnetic resonances and mass spectrum). The spectral data were identical with those previously reported (29). 4-NQO (CAS: 56-57-5, 98% pure) was obtained from Wako Pure Chemical Ind. Co., Ltd., Osaka, Japan. Experimental diets mixed with auraptene at doses of 100 and 500 ppm were weekly prepared, and a feeder was placed in each cage. 4-NQO solution (20 ppm) was prepared on a weekly basis. The diets and 4-NQO solution were stored in a cold room (4°C) until used. They were freely available.

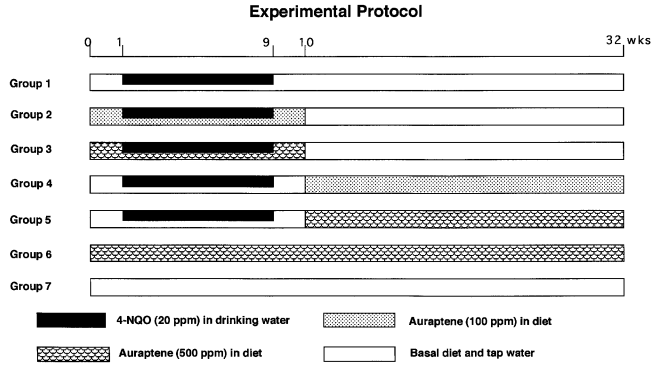

Fig. 2. Experimental protocol.

Experimental procedure

The experiment was designed to examine the modifying effects of auraptene during the initiation and post-initiation phases of 4-NQO-induced oral tumorigenesis in male F344 rats.

A total of 122 rats were divided into seven groups: details of the treatment with 4-NQO and auraptene are shown in Figure 2 and Tables 1–6. At 7 weeks of age, rats in groups 1 through 5 were given 4-NQO (20 ppm) in the drinking water for 8 weeks. Groups 2 and 3 were respectively given the diets containing 100 ppm and 500 ppm auraptene, starting at 6 weeks of age until 1 week after the stop of the carcinogen exposure. They were then switched to the basal diet and maintained on this diet for 22 weeks. Groups 4 and 5 were, respectively, fed the diets mixed with 100 and 500 ppm auraptene, starting 1 week after the cessation of 4-NQO treatment and continued on these diets for 22 weeks. Group 6 were fed the diet containing 500 ppm auraptene during the experiment. Group 7 was given the basal diet and tap water throughout the experiment and served as an untreated control.

All rats were carefully inspected daily. Diet consumption and spillage were monitored three times before and after carcinogen treatment. Water consumption was monitored twice a week during the experiment. The experiment was terminated at 32 weeks and all animals were killed by decapitaion to evaluate the frequencies of preneoplastic and neoplastic lesions in the oral cavity. At necropsy, all organs, especially oral cavity were grossly examined and all organs except for tongue were fixed in 10% buffered formalin. Tongues were cut approximately into two halves; one portion was used for polyamine assay and the other for histopathology and cell proliferation counts. The tongues used for these measurements basically did not contain tumorous lesions. For histopathological confirmation, tissues and gross lesions were fixed in 10% buffered formalin, embedded in paraffin blocks and processed by the conventional histological methods using haematoxylin and eosin stain. Epithelial lesions (hyperplasia, dysplasia and neoplasia) in the oral cavity were diagnosed according to the criteria described by Bánóczy and Csiba (30) and Kramer *et al*. (31).

Determination of BrdU-labelling index in the non-lesional tongue epithelium

To assess the proliferative activity of squamous epithelium of the tongue, BrdU-labelling index of all animals were quantified. For measurement of BrdU-incorporated nuclei, the animals were given an i.p. injection of 50 mg/kg body wt BrdU (Sigma Chem. Co., Ltd., St. Louis, MO) 1 h prior to killing.

Table I. Incidence of tongue dysplasia of rats given 4-NQO together with auraptene (a pilot study)

Treatment	No. of rats with dysplasia	No. of rats with:			
		Mild dysplasia	Moderate dysplasia	Severe dysplasia/cm	No. of dysplasia/cm
4-NQO alone	5/5	5/5	5/5	1/5	0.51 ± 0.15[a]
4-NQO + 100 ppm auraptene	3/5	2/5	3/5	0/5	0.32 ± 0.10[b]
4-NQO + 500 ppm auraptene	3/5	2/5	1/5[c]	0/5	0.22 ± 0.08[d]
No treatment	0/5	0/5	0/5	0/5	

[a]Mean ± SD.
[b,d]Significantly different from group 1 by Student's *t*-test ([b]$P < 0.05$ and [d]$P < 0.01$).
[c]Significantly different from group 1 by Fisher's exact probability test ($P = 0.0238$).

Table II. Body, liver and relative liver wts in each group

Group no.	Treatment	No. of rats examined	Body wt (g)	Liver wt (g)	Relative liver wt (g/100 g body wt)
1	4-NQO alone	21	332±35[a]	11.9±0.9[b]	3.51±0.14[b]
2	4-NQO + auraptene (100 ppm)	19	345±26	13.8±2.3[c]	3.98±0.52[c]
3	4-NQO + auraptene (500 ppm)	19	342±26	14.3±2.1[d]	4.09±0.34[d]
4	4-NQO → auraptene (100 ppm)	17	322±20	12.5±1.8	3.88±0.42[c]
5	4-NQO → auraptene (500 ppm)	20	334±23	13.8±1.7[d]	4.12±0.31[d]
6	Auraptene (500 ppm)	12	346±25	13.3±2.0	3.85±0.55
7	No treatment	14	343±18	13.2±1.2	3.83±0.24

[a]Mean ± SD.
[b]Significantly different from group 7 by Dunnett's *t*-test ($P < 0.001$).
[c,d]Significantly different from group 1 by Dunnett's *t*-test ([c]$P < 0.005$ and [d]$P < 0.001$).

Table III. Incidence of tongue preneoplasms and neoplasms in rats of each group

Group no.	Treatment examined	No. of rats (%)	Hyperplasia (%)	Dysplasia (%)	Papilloma (%)	Squamous cell carcinoma (%)
1	4-NQO alone	21	21 (100)	21 (100)	4 (19)	12 (57)
2	4-NQO + auraptene (100 ppm)	19	19 (100)	19 (100)	1 (5)	1 (5)[a]
3	4-NQO + auraptene (500 ppm)	19	19 (100)	19 (100)	4 (21)	4 (21)[b]
4	4-NQO → auraptene (100 ppm)	17	16 (94)	16 (94)	2 (13)	0 (0)[a]
5	4-NQO → auraptene (500 ppm)	20	20 (100)	20 (100)	1 (5)	3 (15)[c]
6	Auraptene (500 ppm)	12	0	0	0	0
7	No treatment	14	0	0	0	0

[a–c]Significantly different from group 1 by Fisher's exact probability test ([a]$P < 0.001$, [b]$P < 0.05$ and [c]$P < 0.01$).

Table IV. Incidence of tongue preneoplasms and in rats of each group

Group no.	Treatment	Hyperplasia (%)			Dysplasia (%)			
		Total	Simple	Papillary	Total	Mild	Moderate	Severe
1	4-NQO alone	21/21 (100)	21/21 (100)	13/21 (62)	19/21 (10)	21/21 (200)	21/21 (200)	16/21 (76)
2	4-NQO + auraptene (100 ppm)	19/19 (100)	18/19 (95)	11/19 (56)	19/19 (100)	17/19 (89)	13/19 (68)[a]	8/19 (42)[b]
3	4-NQO + auraptene (500 ppm)	19/19 (100)	18/19 (95)	8/19 (42)	19/19 (100)	19/19 (00)	10/19 (53)[c]	10/19 (53)
4	4-NQO → auraptene (100 ppm)	16/17 (94)	16/17 (94)	11/17 (65)	16/17 (94)	16/17 (94)	12/17 (71)[b]	6/17 (35)[b]
5	4-NQO → auraptene (500 ppm)	20/20 (100)	20/20 (100)	11/20 (55)	20/20 (100)	18/20 (90)	18/20 (90)	8/20 (40)[b]
6	Auraptene (500 ppm)	0/12 (0)	0/12 (0)	0/12 (0)	0/12 (0)	0/12 (0)	0/12 (0)	0/12 (0)
7	No treatment	0/14 (0)	0/14 (0)	0/14 (0)	0/14 (0)	0/14 (0)	0/14 (0)	0/14 (0)

[a–c]Significantly different from group 1 by Fisher's exact probability test or Chi-square test ([a]$P < 0.01$, [b]$P < 0.05$ and [c]$P < 0.001$).

Table V. Polyamine levels of tongue of rats in each group

Group no.	Treatment	No. of rats examined	Polyamine levels (nmol/mg protein)			
			Diamine	Spermidine	Spermine	Total
1	4-NQO alone	21	0.26 ± 0.25[a,b]	1.82 ± 0.73	1.51 ± 0.30	3.58 ± 0.41
2	4-NQO + auraptene (100 ppm)	19	0.14 ± 0.12	1.56 ± 0.33	1.53 ± 0.20	3.23 ± 0.36[c]
3	4-NQO + auraptene (500 ppm)	19	0.12 ± 0.11[d]	1.54 ± 0.34	1.53 ± 0.21	3.19 ± 0.36[e]
4	4-NQO → auraptene (100 ppm)	17	0.15 ± 0.20	1.58 ± 0.44	1.62 ± 0.27	3.35 ± 0.50
5	4-NQO → auraptene (500 ppm)	20	0.08 ± 0 16	1.55 ± 0.33	1.51 ± 0.20	3.14 ± 0.45[e]
6	Auraptene (500 ppm)	12	0.07 ± 0.12	1.62 ± 0.36	1.54 ± 0.19	3.23 ± 0.34
7	No treatment	14	0.06 ± 0.09	1.59 ± 0.36	1.64 ± 0.28	3.28 ± 0.53

[a]Mean ± SD.
[b]Significantly different from group 7 by Dunnett's test ($P < 0.01$).
[c–e]Significantly different from group 1 by Dunnett's test ([c]$P < 0.01$, [d]$P < 0.05$ and [e]$P < 0.005$).

Table VI. BrdU-labelling index of non-lesional area of tongue squamous epithelium

Group no.	Treatment	No. of rats examined	BrdU-labelling index (%)
1	4-NQO alone	21	6.79 ± 0.90[a,b]
2	4-NQO + auraptene (100 ppm)	19	6.15 ± 0.94[c]
3	4-NQO + auraptene (500 ppm)	19	5.97 ± 0.91[d]
4	4-NQO → auraptene (100 ppm)	17	5.95 ± 0.63[e]
5	4-NQO → auraptene (500 ppm)	20	5.39 ± 1.29[f]
6	Auraptene (500 ppm)	12	5.45 ± 0.91
7	No treatment	14	5.23 ± 0.78

[a]Mean ± SD.
[b]Significantly different from group 7 by Dunnett's test ($P < 0.001$).
[c–f]Significantly different from group 1 by Dunnett's test ([c]$P < 0.05$, [d]$P < 0.01$, [e]$P < 0.005$, and [f]$P < 0.001$).

The tongue was removed and cut into two. One-half was used for polyamine assay and the other was fixed in 10% buffered formalin for histopathology and BrdU-labelling index measurement. Two serial sections (3 μm in thickness) were made after embedding in paraffin. The one section was used for immunohistochemical detection of BrdU incorporation using an immunohistochemical analysis kit (Amersham, UK). The labelling indices of BrdU (%) were calculated by counting for labelled nuclei of 100 cells from each rat under ×400 magnification. The other section was used for histopathological diagnosis.

Polyamine levels of tongue tissue

The polyamines in the oral cavity tissues were measured by the method of Koide *et al*. (32). The results obtained were confirmed to be correlated well with those by high performance liquid chromatography. At death, one-half of the tongues of all rats were collected and the amounts of diamine, spermine and spermidine were determined by an enzymatic differential assay.

GST and QR assay

Twenty-five rats aged 5 weeks were used for GST and QR assay. They daily received various doses of auraptene (0, 200, 400 or 800 mg/kg body wt) in 0.5 ml of 5% gum arabic (Sigma Chemical Co.) by gavage for 5 days. They were killed by cervical dislocation at 30 min after the last gavage of auraptene. At death, the liver and tongue were excised immediately. The liver was perfused with saline to remove blood and minced into small pieces. The tongue was slit longitudinally and washed with phosphate-buffered saline (pH 7.4) and the mucosa was collected by scraping the mucosal surface using a microtome knife. Aliquots of minced liver and mucosal scrapings were processed for cytosolic fraction as described (33,34). The activities of GST with 1-chloro-2,4-dinitrobenzene (CDNB) and 1,2-dichloro-4-nitrobenzene (DCNB) as substrates and QR with NADH and menadione as substrates were determined as described previously (35–37). All assays were performed by spectrophotometry at 340 nm and all samples were measured in quadruplicate. One unit of enzyme activity is the amount of enzyme catalysing the conversion of 1 μmol substrate to product per min at 25°C. Cytosolic protein concentrations were determined by the Bradford method (38) using bovine serum albumin as the standard.

Statistical analysis

Statistical analysis on the incidence of lesions was performed using Fisher's exact probability test or Chi-square test, and the data of measurements of body wt, liver wt, polyamine assay, BrdU-labelling index, and GST and QR assay were compared by Student's *t*-test or Dunnett's *t*-test. The results were considered statistically significant if the *P* value was 0.05 or less.

Results

Incidence of dysplasia in a pilot study

The results of a pilot study are summarized in Table I. The incidence of moderate dysplasia in rats treated with 4-NQO and 500 ppm auraptene (one out of five rats) was significantly lower than that of rat given 4-NQO alone (five out of five rats; $P = 0.0238$). The mean number of dysplastic lesions per cm of tongue epithelium in rats given 4-NQO and 100 or 500 ppm auraptene was significantly smaller than that of 4-NQO ($P < 0.05$ or < 0.01).

General observations

Rats in groups 1–6 tolerated well the oral administration of 4-NQO and/or auraptene. There were no significant differences on a mean intake of 4-NQO/day/rat among five groups (groups 1–5: 0.220–0.231 mg). The mean body and liver wts at the end of the study are indicated in Table 2. The mean body wt of rats did not significantly differ among the groups. The mean liver wt of animals in group 1 (4-NQO alone) was significantly smaller than that of group 7 ($P < 0.001$). The average liver wts of animals in groups 2 (4-NQO + 100 ppm auraptene), 3 (4-NQO + 500 ppm auraptene) and 5 (4-NQO → 500 ppm auraptene) were significantly greater than that of group 1 ($P < 0.005$ or $P < 0.001$). The average relative liver wt (g/100 g body wt) of group 1 was significantly smaller than that of group 7 ($P < 0.001$) and the values of groups 2 through 5 were significantly larger than that of group 1 ($P < 0.005$ or < 0.001).

Incidences of tumours and preneoplastic lesions

In the present study, endophytic or exophytic tumours occurred only in the dorsal site of the tongue of rats in groups 1–5: the former being microscopically well-differentiated squamous cell carcinoma and the latter squamous cell papilloma. The incidences of tongue neoplasms (squamous cell papilloma and carcinoma) in each group are shown in Table III. In group 1 (4-NQO alone), the incidences of tongue squamous cell carcinoma and squamous cell papilloma were 57% (12/21 rats) and 19% (4/21 rats), respectively. On the other hand, only a few rats given auraptene during or after 4-NQO administration had

tongue neoplasms (papilloma: 5% in group 2, 21% in group 3, 13% in groups 4, 5% in group 5; carcinoma: 5% in group 2, 21% in group 3, 0% in group 4, 15% in group 5). The incidences of squamous cell carcinoma in groups 2–5 were significantly smaller than that of group 1 ($P < 0.001$, < 0.05 or < 0.01). No such neoplasms developed in animals of groups 6 and 7.

In addition to these neoplasms, a number of hyperplasia and dysplasia that are considered to be preneoplastic lesions for oral cancer were present in the tongue of rats in groups 1 through 5, but not in rats of groups 6 and 7. The incidences of such lesions are also listed in Table IV. Almost all rats in groups 1–5 had hyperplasia and dysplasia in their tongue: their incidences being 100 or 94% (Table II). Hyperplasia could be classified into two categories simple hyperplasia and papillary hyperplasia, and three types of dysplasia (mild, moderate and severe dysplasia) according to the degree of atypism. The incidences of such hyperplasia and dysplasia are summarized in Table III. In rats of group 1, the incidence of simple hyperplasia was 100% and papillary hyperplasia 62%. In rats of groups 2–5, the incidence of papillary hyperplasia was smaller than group 1 without statistical differences. Rats in group 1 had 100% incidence of mild dysplasia, 100% with moderate dysplasia and 76% severe dysplasia. The frequencies of moderate dysplasia in groups 2–4 (53–71%), and those of severe dysplasia in groups 2, 4, 5 and 7 (35–47%) were significantly lower than that of group 1 ($P < 0.05$, < 0.01 or < 0.001).

Polyamine levels

The results of polyamine assay of tongue mucosa are shown in Table V. The average diamine level in rats of group 1 was significantly greater than that of group 7 ($P < 0.01$). The mean diamine level of group 3 was significantly smaller than that of group 1 ($P < 0.05$). The mean amounts of total polyamine levels (diamine + spermidine + spermine) in groups 2, 3 and 5 were significantly lower than that of group 1 ($P < 0.01$ or < 0.005). Those values in group 6 were almost comparable to those in group 7.

BrdU-labelling index

The results of morphometric analysis BrdU-labelling indices in the non-lesional tongue squamous epithelium are summarized in Table 6. The mean index in the non-lesional tongue epithelium exposed to 4-NQO alone (group 1) was significantly larger than those of untreated control (group 7) ($P < 0.001$). Dietary administration of auraptene during initiation or post-initiation (groups 2–5) significantly decreased the indices ($P < 0.05$, < 0.01, < 0.005 or < 0.001). The average BrdU-labelling indices in groups 6 and 7 was almost similar.

Activities of GST and QR in liver and tongue

The data of GST and QR assays in the liver and tongue are illustrated in Figures 3 and 4. Oral exposure of auraptene could modulate these enzyme levels in both organs. As shown in Figure 3a,b, liver GST activity was significantly increased by gavage of auraptene at doses of 400 and 800 mg/kg body wt (1.54- and 1.63-fold increase for GST-CDNB, and 1.46-fold increase for GST-DCNB, $P < 0.005$ to < 0.001). Similarly, auraptene administration (200, 400 and 800 mg/kg body wt) significantly elevated liver QR activity (1.69-, 1.88- and 2.35-fold increase, $P < 0.005$ or $P < 0.001$, Figure 3c). In the tongue mucosa, significantly increased levels of GST (at doses of 200, 400 and 800 mg/kg body wt) and QR (at doses of 400 and 800 mg/kg body wt) were found (1.28-, 1.32- and 1.43-fold increase for GST-CDNB, and 1.17- and 1.38-fold increase for QR, $P < 0.05$ to < 0.001), as indicated in Figure 4a,b.

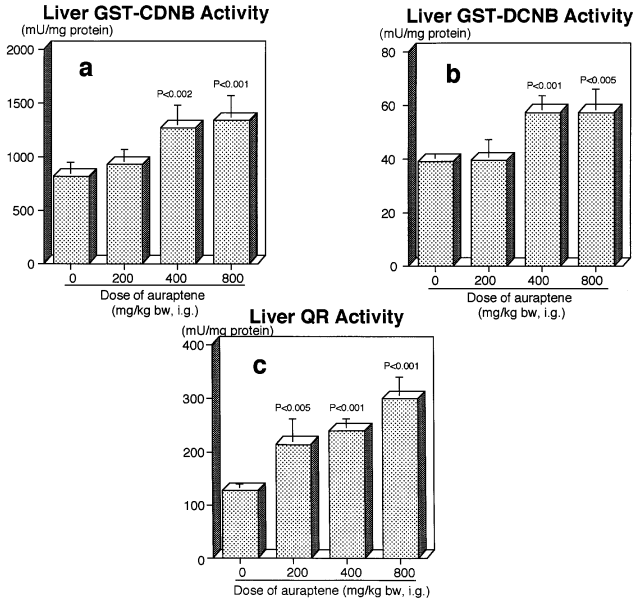

Fig. 3. Effect of auraptene on GST and QR activities in liver. (**a**) GST-CDNB activity, (**b**) GST-DCNB activity and (**c**) QR activity in rats daily gavaged 0, 200, 400 or 800 mg auraptene/kg body wt for 5 days.

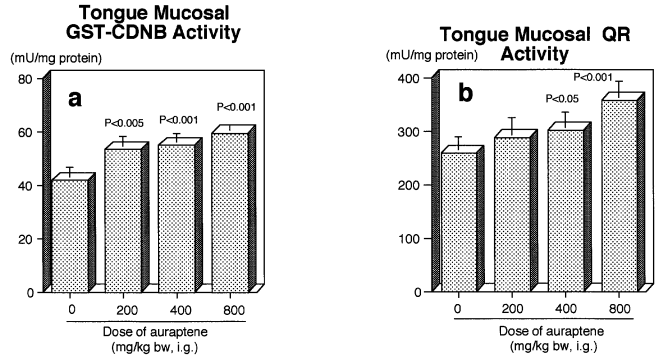

Fig. 4. Effect of auraptene on GST and QR activities in tongue mucosa. (**a**) GST-CDNB activity and (**b**) QR activity in rats daily gavaged 0, 200, 400 or 800 mg auraptene/kg body wt for 5 days.

Discussion

The results in the present study demonstrated that auraptene feeding during either the initiation or post-initiation phase effectively suppressed 4-NQO-induced oral carcinogenesis as revealed by reduction in the incidence of tongue carcinoma. Feeding of the diets mixed with auraptene also suppressed the cell proliferation biomarker's expression, BrdU-labelling index and polyamine level in the tongue mucosa. In addition, auraptene elevated the activities of GST and QR in the liver and tongue. In the present study, the results in a long-term study confirmed those in a pilot study using the incidence of dysplasia as a biomarker. This may suggest that such a short-term pilot study may be useful for detecting compounds possessing 'blocking (39)' chemopreventive property against oral carcinoma.

Vegetable and fruits contain a variety of compounds that inhibit mutagenesis and/or carcinogenesis in laboratory animals

(40). These include limonoids (*d*-limonene) (21,41,42) and flavonoids (hesperidine and diosmin) (15,17,24,43). Certain types of coumarin found in various foods (44) are potent anti-tumour promoters (45). An area of considerable importance in the identification of chemopreventive agents for development for possible human use pertains to toxicity. In the current study, a coumarin-related compound auraptene present in citrus fruits and their processed juices (26) did not show any cytotoxicity, as found in previous studies (26,27). The anti-platelet action of auraptene (25) did not occur at dose levels used in the present and previous studies (26,27). Thus, it is likely that auraptene is a new member of chemopreventive compounds from citrus fruits and is applicable for human clinical trials, although no or less toxicity of the compound should be confirmed by the 2-year bioassay.

The results of the present study indicate that dietary administration of auraptene at the dose levels of 100 and 500 ppm during two different stages of 4-NQO-induced oral tumorigenesis could effectively suppress tumour development. In contrast to the dose-dependent preventive effect of auraptene on aberrant crypt foci development (27), the low dose (100 ppm) of auraptene was more effective: the post-initiation feeding of 100 ppm auraptene completely inhibited tongue squamous cell carcinoma. The reasons of this discrepancy are not known, although the experimental conditions, such as the carcinogens used and their target organs were different. Additional studies concerning tissue distribution of this compound, etc., are needed, but the data suggest the evaluation of lower doses of this compound.

Several mechanisms by which auraptene exerts its anti-carcinogenic action in oral carcinogenesis could be considered. Although prevention of carcinogenesis might be due to multiple mechanisms, one way of action of anticarcinogens is an enhancing effect on carcinogen detoxification systems. Fruits and vegetables that elevate tissue phase II enzyme levels in rodents can effectively block experimental carcinogenesis and increase the clearance of drugs in human (10,11,46). It is reported that coumarin-related compounds exert anti-carcinogenic effects through modulation of phase II enzymes and their conjugation with electrophilic forms of carcinogenic metabolites (47,48). In the current study, auraptene significantly induced GST and QR activities in the liver and tongue. In our previous study, auraptene was found to elevate the GST and QR activities in the colon (27). Like some chemopreventive agents (28,39), therefore, auraptene administered during the initiation phase could exerts its chemopreventive action by enhancing carcinogen detoxification systems, such as GST and QR, although it is not known which classes (alpha, mu, pi and/or theca) of GSTs auraptene affects.

Cell proliferation is suggested to play an important role in multistage carcinogenesis (especially during the post-initiation or promotion phase), including oral tumorigenesis (12,49–51). In this study, auraptene inhibited polyamine levels and cell proliferation activity (BrdU-labelling index) of tongue mucosa induced by 4-NQO exposure. These results were comparable to our earlier experiments, demonstrating the chemopreventive efficacy of several natural antioxidants (12) and some synthetic compounds (52,53). In rodents and human oral carcinogenesis, increased polyamine levels and/or ornithine decarboxylase (ODC) activity that are essential for cellular proliferation were reported (54). It is also known that agents which inhibit ODC activity or polyamine levels are effective tumour inhibitors (49,54,55). Thus, suppressing effects of auraptene administered during the post-initiation stage might be due to suppression of cell proliferation sensitivity. Other possible mechanisms, including antioxidant property (12,49) are also considered, since auraptene is reported to suppress superoxide (O_2^-) generation induced by a promoter 12-*O*-tetradecanoylphorbol-13-acetate, and suspected to block the assembly of upstream signal transduction systems involved in the activation of the NADPH oxidase system (26). Such an effect may also contribute the protective effect of auraptene when fed during the post-initiation phase of carcinogenesis.

In summary, dietary administration of auraptene significantly inhibited 4-NQO-induced tongue tumorigenesis, in conjunction with reduction in the frequency of dysplastic lesions, inbition of cell proliferation biomarker's expression and induction of phase II enzymes GST and QR in the liver and tongue. Our findings, together with previous reports (26,27) show that auraptene is a chemopreventive agent that possesses anticarcinogenic potency in tumorigenesis of multiple organ (skin, colon and oral cavity).

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