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Effect of α-naphthyl isothiocyanate on 2-amino-3-methylimidazo[4,5-*b*]pyridine (PhIP)-induced mammary carcinogenesis in rats

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The modifying effects of α-naphthyl isothiocyanate (ANIT) on 2-amino-3-methylimidazo[4,5-*b*]pyridine (PhIP)-induced mammary carcinogenesis were investigated in female Sprague-Dawley (SD) rats, and the hepatic activities of the phase II detoxifying enzymes glutathione *S*-transferase (GST) and quinone reductase (QR) were also assayed. Ninety-eight rats were divided into 4 groups. Starting at 6 weeks of age, rats were fed the high-fat diet without ANIT (Groups 1 and 4) or the experimental diet (high-fat diet mixed with 400 ppm ANIT, Groups 2 and 3). At 7 weeks of age, Groups 1 and 2 were given PhIP in corn oil (85 mg/kg body weight, 8 times for 11 days) by intragastric intubation. One week after the last PhIP injection, 5 rats in each group were sacrificed to assay GST and QR activities, and the experimental diets for Groups 2 and 3 were switched to the high-fat diet without ANIT until termination of the experiment. Group 4 served as the vehicle control. All rats were sacrificed at 24 weeks after the start of the experiment. At termination of the experiment, mammary tumours were detected in Groups 1 (PhIP alone) and 2 (PhIP + ANIT) and were shown histologically to be adenocarcinomas; their incidences (multiplicities) were 56.3% (1.66 ± 2.31/rat) in Group 1 and 6.7% (0.07 ± 0.25/rat) in Group 2 ($p < 0.001$). Mean sizes of the tumours were 10.6 ± 5.3 mm in Group 1 and 6.5 mm in Group 2. No mammary tumours were observed in rats of Groups 3 and 4. In addition, ANIT treatment significantly increased the activities of GST and QR in the livers of rats in Groups 2 and 3 as compared to Groups 1 and 4. These results imply that the isothiocyanate compound ANIT shows potent inhibitory effects on mammary carcinogenesis induced by PhIP in female SD rats when administered during the initiation stage.

Key words: PhIP; α-naphthyl isothiocyanate; mammary carcinogenesis; chemoprevention; rat

Among a number of environmental factors, dietary habits have been regarded as the most important determinant for cancer development in humans.[1–3] Epidemiological data suggest that individuals eating fried or broiled meat have a significantly elevated risk of intestinal cancer.[4,5] A number of carcinogenic heterocyclic amines (HCAs) have been detected in cooked foods. These HCAs show potent mutagenicity in the Ames test and their carcinogenic potential has been demonstrated in various organs, including the forestomach, liver, small intestine, large intestine, mammary gland, Zymbal's gland, clitoral glands, haematopoietic systems, blood vessels and skin of rodents.[6,7] For example, 2-amino-3-methylimidazo[4,5-*b*]pyridine (PhIP) has attracted considerable attention for potent mutagenicity and carcinogenic potential in the mammary gland and colon of rats.[8,9] In most previous studies of their carcinogenesis, the long-term administration of HCAs in the diet has been needed for the development of tumours.[10] In rat mammary carcinogenesis induced by PhIP, mammary cancers are induced at a rate of 47% by administration of 0.04% PhIP in the diet for 52 weeks.[8] Ghoshal *et al.*[11] found mammary tumours in 53% of Sprague-Dawley (SD) rats fed a high-fat diet 25 weeks after treatment with PhIP in 10 doses of 75 mg/kg body weight for 12 days. It was also reported that mammary tumours developed in 77% of CD rats 41 weeks after treatment with PhIP at 50 μmol/rat/week for 8 weeks on a high-fat diet.[12] In these studies, a high-fat diet was given throughout the experiment. These results indicate that the development of PhIP-induced mammary tumours is enhanced and induced earlier by feeding with a high-fat diet. On the other hand, a high-fat diet did not affect PhIP-induced mammary carcinogenesis in SD × F344 F_1 hybrid rats.[13]

α-Naphthyl isothiocyanate (ANIT), present as the glucosinolate precursor, is a constituent of cruciferous vegetables.[14] ANIT has been reported to induce cholestasis, bile duct proliferation and focal necrosis of hepatocytes, without the development of liver cancer, and to inhibit hepatocarcinogenesis in rats.[15–17] The mechanisms of its chemopreventive effect have been discussed;[18] one possible mechanism is the regulation of phase I and phase II enzymes. ANIT has been shown to decrease hepatic cytochrome P-450 content,[19,20] and hepatic mixed-function oxidase activities,[21] and increase microsomal epoxide hydrolase and cytosolic DT-diaphorase activities.[19] The promutagenic PhIP found in cooked foods is converted to the active form mainly by cytochromes P450 1A1, 1A2 and 1B1 in the liver and partially in the target organs.[21–27] Glutathione *S*-transferase (GST) has been reported to inhibit DNA binding of *N*-acetyl-PhIP *in vitro*.[28]

These findings suggested that the administration of P450 inhibitor in the initiation phase may reduce the carcinogenicity of PhIP. ANIT was used in early studies of cancer chemoprevention, although it is toxic to the liver and is a cholestatic agent.[15–17] Possibly as a result of this, it is now used rarely in chemoprevention studies and has not been scheduled for trials in humans. Toxicity is a serious problem for trials of cancer chemopreventive agents. However, if lower doses of such compounds do not exert clear toxicity in any organs, including the liver, these compounds should not be omitted from basic research on cancer chemoprevention.

In our study, we examined the effects of a low dose (400 ppm in diet) of ANIT on PhIP-induced mammary carcinogenesis in SD rats. In addition, the activities of phase II detoxifying GST and quinone reductase (QR) in the liver were measured to clarify whether these enzymes are involved in its modification of PhIP-induced mammary carcinogenesis.

Material and methods

Animals, diet, water and carcinogen

Weanling female SD rats were purchased from Japan SLC, Co. (Hamamatsu, Japan). PhIP was supplied by Dr. Wakabayashi

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TABLE I – PERCENT COMPOSITION OF EXPERIMENTAL SEMIPURIFIED HIGH FAT DIETS CONTAINING CORN OIL

Diet ingredients	Percents of ingredients by weight
Casein, vitamin-free	23.50
DL-Methionine	0.35
Corn starch	32.90
Dextrose	8.30
Alphacel	5.90
Corn oil	23.52
Mineral, AIN	4.11
Vitamin, AIN (revised)	1.18
Choline bitartrate	0.24

AIN: American Institute of Nutrition.

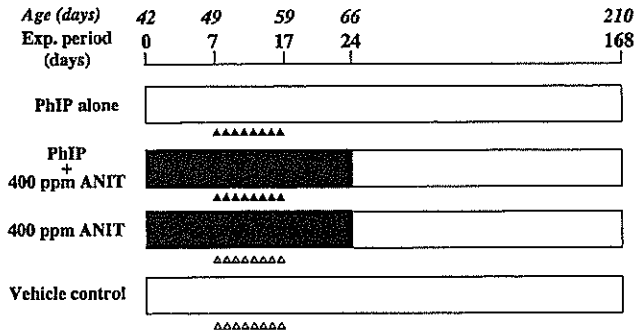

FIGURE 1 – Experimental protocol. Unshaded, high-fat diet; shaded, high-fat diet with 400 ppm α-naphthyl isothiocyanate (ANIT). Closed triangle, PhIP 85 mg/kg B.W. in corn oil by gavage Open triangle, Corn oil by gavage.

(National Cancer Center Research Institute, Tokyo, Japan). ANIT was purchased from Tokyo Kasei Kogyo Co., Ltd. (Tokyo, Japan). All diet ingredients were obtained from CLEA Japan Inc. (Tokyo, Japan) and experimental and high-fat diets were prepared weekly in our laboratory and stored in a cold room (<4°C). The composition of the high-fat diet is shown in Table I. All animals were housed in wire cages (3 rats/cage). Animals had free access to water and diets under controlled environmental conditions of humidity (50 ± 10%), lighting (12 hr light/dark cycle) and temperature (23 ± 2°C).

Animal treatment and pathological examination

A total of 98 rats, 5 weeks of age, were divided into 4 groups: Group 1, 37 rats given PhIP alone; Group 2, 35 rats given PhIP and 400 ppm ANIT in the initiation phase; Group 3, 13 rats given ANIT alone and Group 4, 13 rats as vehicle controls. All animals were given the high-fat diet throughout the experiment as the basal diet (Fig. 1).

Rats in Groups 2 and 3 were given diet containing 400 ppm ANIT from the start of the experiment and animals in the other groups were kept on the basal diet. Animals in Groups 1 and 2 were given 8 doses of PhIP (85 mg/kg body weight, gastric intubation) in 0.1 ml of corn oil for 11 days, starting 7 days after the commencement of the experiment. Vehicle control rats received an equal volume of corn oil. For rats in Groups 2 and 3, the experimental diet was changed to the basal diet 1 week after final treatment with the carcinogen. Five rats in each group were sacrificed to assay GST and QR activities at 1 week after the last PhIP injection. All other animals were sacrificed 24 weeks after the start of the experiment to assess the modifying effect of ANIT on mammary carcinogenesis induced by PhIP. At the end of the experiment, complete autopsies on these animals were performed after sacrifice by ether anaesthesia. At autopsy, the location, number

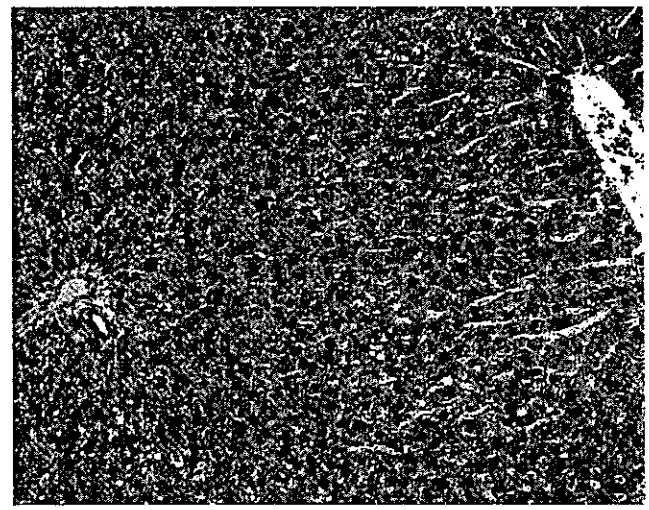

FIGURE 2 – Histology of liver of a rat from Group 3 (ANIT alone) reveals no abnormalities. Hematoxylin and eosin stain, ×10 (original magnification).

and size of mammary tumours were recorded. Tissues were fixed in 10% buffered formalin, embedded in paraffin blocks and processed for routine histological observation with haematoxylin and eosin staining. Pathological diagnosis of mammary tumours was performed according to the criteria outlined by Young and Hallowes.[29]

GST and QR assays

Aliquots of minced liver were processed to obtain the cytosolic fraction. The activities of GST and QR were determined using 1-chloro-2,4-dinitrobenzene (CDNB) or 1,2-dichloro-4-nitrobenzene (DCNB) for GST and NADH/menadione for QR as substrates, respectively, as described previously.[30–32] All spectrographic assays were based on absorption at 340 nm and all samples were examined in triplicate. One unit of enzyme activity was defined as the amount of enzyme catalysing the conversion of 1 μmol of substrate to product per min at 25°C. Cytosolic protein concentrations were determined by the Bradford method[33] using bovine serum albumin as the standard.

Statistical analysis

Differences in the incidence or multiplicity of mammary tumours between groups were analysed by Fisher's exact probability test, χ^2-test, Student's t-test or alternate Welch t-test.

Results

Table I shows body, liver and relative liver weights of rats in each group at the termination of the experiment. No significant differences were found in body or liver weights between Groups 1 (PhIP alone) and 2 (PhIP + ANIT). Body weight of Group 3 (ANIT alone) was significantly lower than that of Group 4 (vehicle control) ($p < 0.005$). Relative liver weight of rats in Group 3 (ANIT alone) was significantly greater than that in Group 4 (vehicle control) ($p < 0.005$). However, no clinical signs of toxicity of ANIT were noted during the study. Histological examination did not provide evidence of histological alterations, including cholestasis, bile duct proliferation or cell necrosis, in the livers of rats given ANIT alone (Fig. 2).

At the end of the experiment, mammary tumours were found in rats in Groups 1 and 2 but no tumours were found in animals of Groups 3 or 4 rats, as summarised in Table II. All of the mammary tumours were diagnosed as invasive or intraductal adenocarcino-

TABLE II – FINAL BODY AND LIVER WEIGHTS

Group	Treatment	Number of rats	Body weight (g)	Liver weight (g)	Relative liver weight (%)
1	PhIP alone	32	278 ± 20[1,2]	11.0 ± 2.4	3.94 ± 0.76
2	PhIP + ANIT	30	277 ± 19	10.3 ± 1.5	3.72 ± 0.52
3	ANIT alone	8	266 ± 19[2]	10.5 ± 1.1	3.94 ± 0.25[2]
4	No treatment	8	304 ± 20	10.4 ± 1.2	3.41 ± 0.30

[1]Mean ± SD.–[2]Significantly different from Group 4 by Student's *t*-test (b: $p < 0.005$).

TABLE III – INCIDENCE AND MULTIPLICITY OF MAMMARY CARCINOMA

Group	Treatment	Number of rats	Incidence (%)			Multiplicity (number/rat)		
			Total	Inv.d.Ca.[1]	Int.d.Ca.[2]	Total	Inv.d.Ca.	Int.d.Ca.
1	PhIP	32	18 (56.3)	17 (53.1)	8 (25.0)	1.66 ± 2.31[3]	1.19 ± 1.64	0.47 ± 1.29
2	PhIP + ANIT	30	2 (6.7)[4]	2 (6.7)[4]	0[5]	0.07 ± 0.25[6]	0.07 ± 0.25[6]	0
3	ANIT alone	8	0	0	0	0	0	0
4	Vehicle control	8	0	0	0	0	0	0

[1]Invasive ductal carcinoma.–[2]Intraductal carcinoma.–[3]a: Mean ± SD.–[4,5]Significantly different from Group 1 (PhIP alone) by Fisher's exact probability test (b: $p < 0.001$, c: $p < 0.005$).–[6]d: Significantly different from Group 1 (PhIP alone) by Student's *t*-test or alternate Welch *t*-test ($p < 0.001$).

TABLE IV – RESULTS OF LIVER GST AND QR ACTIVITIES

Group	Treatment	Number of rats	GST		QR
			CDNB (mU/mg)	DCNB (mU/mg)	NADH (mU/mg)
1	PhIP alone	5	415.6 ± 76.1[1,2]	8.41 ± 0.17[3]	126.7 ± 28.6[4]
2	PhIP + ANIT	5	491.0 ± 145.0	21.29 ± 0.44[5]	222.6 ± 2.2[6]
3	ANIT alone	5	322.4 ± 72.6	17.63 ± 0.39[3]	234.4 ± 94.5[7]
4	No treatment	5	269.0 ± 87.0	4.82 ± 0.87	61.4 ± 7.7

[1]Mean ± SD.–[2,3,4,7]Significantly different from Group 4 (No treatment) by Student's *t*-test or alternate Welch *t*-test (b: $p < 0.05$, c: $p < 0.001$, d: $p < 0.005$, g: $p < 0.02$).–[5,6]Significantly different from Group 1 (PhIP alone) by Student's *t*-test or alternate Welch *t*-test (e: $p < 0.001$, f: $p < 0.002$).

mas. The incidence of mammary adenocarcinoma in Group 2 (6.7%, $p < 0.001$) was significantly lower than that in Group 1 (56.3%). The average number of neoplasms in Group 2 (0.07 ± 0.25, $p < 0.001$) was also significantly lower than that in Group 1 (1.66 ± 2.31). Mean size of mammary tumours in Group 2 (6.5 mm) was smaller than that in Group 1 (10.6 ± 5.3 mm). No neoplastic lesions other than mammary tumours were observed in rats in any group.

Data regarding liver GST and QR activities as determined by short-term bioassay are summarised in Table III. GST activities toward CDNB in Group 1 was slightly but significantly ($p < 0.05$) higher than that in Group 4, whereas that in Group 2 was not significantly different from that in Group 1. GST activities toward DCNB in Groups 1 and 3 were significantly higher than that in Group 4 ($p < 0.001$). Combined treatment with PhIP and ANIT (Group 2) significantly increased GST-PCNB as compared to Group 1 treated with PhIP alone ($p < 0.001$) and showed a summation effect on induction of this activity. Liver QR activities in Groups 1 and 3 were significantly greater than that in Group 4 ($p < 0.005$). Combined treatment with PhIP and ANIT significantly increased QR activity ($p < 0.002$) but did not show a summation effect.

Discussion

In our study, a high incidence of mammary tumours was observed in rats given PhIP and a high-fat diet. It has been reported that long-term administration of HCAs is needed for the development of tumours in rodents.[8,11,12] We observed a higher incidence of mammary tumours as compared to the results reported previously by Ghoshal *et al.*[11] and El-Bayoumy *et al.*[12] Ghoshal *et al.*[11] reported that daily doses of 100 mg/kg of PhIP were toxic to the animals after the 7th dose and that weight loss was seen in surviving animals. In this experiment, 8 doses of 85 mg/kg PhIP were administered to rats for 11 days and did not cause body weight loss.

Treatment with ANIT alone in our study reduced the body weight as compared to that in vehicle-only controls. ANIT is known to induce hepatotoxicity and cholestasis in rats.[15–17] The dose (400 ppm in diet) of ANIT in our study was too low to induce liver toxicity or cholestasis and was about half the dose required to induce hepatotoxicity in rats. In fact, histological examination revealed neither hepatotoxicity nor cholestasis in rats given AINT. The experimental schedule (initiation feeding with ANIT) may be related to the lack of liver toxicity seen in our study.

Several chemopreventive agents for PhIP-induced mammary carcinogenesis, including synthetic or natural compounds with antioxidative properties, have been reported.[34–41] Their cancer chemopreventive effects were mostly observed when given during the entire experimental period or in the post-initiation phase of PhIP-induced mammary tumorigenesis, although diallyl disulfide and aspirin inhibited PhIP-induced mammary carcinogenesis when administered during the initiation phase.[34]

A number of mechanisms underlying chemoprevention by xenobiotics in PhIP-induced mammary tumorigenesis have been proposed. PhIP is oxidised to an *N*-hydroxy derivative [PhIP (NHOH)] in the liver by cytochrome P450 enzymes, and esterified by acetyltransferases or sulfotransferases to the ultimate carcinogen.[42–45] Adduct formation is thought to be crucial for PhIP-induced carcinogenesis and PhIP-DNA adducts have been detected in human tissues.[46] Certain isothiocyanates (*e.g.*, sulphoraphene, phenyl isothiocyanate and benzyl isothiocyanate (BITC)) have been reported to prevent chemically induced cancer development in laboratory animals.[47–55] Isothiocyanates exert their cancer chemopreventive action by modulating the activities of phase I and phase II drug metabolism enzymes.[47–53,55] ANIT can decrease hepatic cytochrome P-450 content.[16–18] PhIP is known to exert its carcinogenic

activity after metabolic activation by CYP1A2 or CYP1B1 mainly in the liver.[21–27] ANIT has been reported to inhibit carcinogenesis in the lung, liver, forestomach, intestine and mammary gland when given before or during carcinogen treatment.[15–18] In our study, ANIT elevated the activities of phase II enzymes, GST and QR in the liver. ANIT has been reported to increase the activity of GST,[20] which can inhibit DNA binding of *N*-acetyl-PhIP *in vitro*.[28] Thus, increased activities of GST and QR, particularly GST, in the liver may be one of the major causes of its cancer-suppressing effect observed in the present study. In our previous experiment, BITC failed to inhibit PhIP-induced mammary carcinogenesis in rats given 100 ppm of PhIP,[56] a higher dose than that used in the present study. The difference between the results of our previous and present studies may be due to the differences in enzyme induction capability of both compounds, BITC and ANIT; BITC shows weaker induction of enzymes involving oxidative metabolism and metabolic conversion than ANIT.[57]

In conclusion, ANIT significantly inhibited breast cancer development induced by PhIP, presumably through the induction of both GST and QR in the liver. Accordingly, ANIT may be a candidate chemopreventive agent for breast cancer. In relation to fat and PhIP, further studies of the effects on levels of CYP proteins, metabolic activation of PhIP by CYP isoforms and another typical phase II enzyme, UDP-glucuronyltrasferase, are necessary to confirm the suppression by ANIT in PhIP-induced mammary carcinogenesis, as UDP-glucuronyltransferase has also been reported to detoxify another heterocyclic amine, 2-amino-3-methylimidazo[4,5-*f*]quinoline (IQ).[58]

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Dietary garcinol inhibits 4-nitroquinoline 1-oxide-induced tongue carcinogenesis in rats

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Abstract

The effects of dietary feeding with a polyisoprenylated benzophenone, garcinol, isolated from *Garcinia indica* fruit rind on the development of 4-nitroquinoline 1-oxide (4-NQO)-induced oral carcinogenesis were investigated in male F344 rats. At 7 weeks of age, animals were given 4-NQO at 20 ppm in the drinking water for 8 weeks to induce tongue neoplasms. They also received the diets containing 100 or 500 ppm garcinol either during (for 10 weeks) or after (for 22 weeks) the carcinogen exposure. The other rats were given tap water without 4-NQO throughout the experiment, and fed garcinol (500 ppm)-containing diet or basal diet alone. At the end of the study (week 32), incidences of tongue neoplasms and preneoplastic lesions, cell proliferation activity in the normal-like tongue epithelium estimated by 5-bromodeoxyurideine (BrdU)-labeling index and cyclin D1-positive cell ratio, and immunohistochemical expression of cyclooxygenase-2 (COX-2) in the tongue lesions were determined. Dietary garcinol significantly decreased the incidence and multiplicity of 4-NQO-induced tongue neoplasms and/or preneoplasms as compared to the control diet. Dietary administration of garcinol also significantly reduced the BrdU-labeling index and cyclin D1-positive cell ratio, suggesting reduction in cell proliferation activity in the tongue by garcinol. The COX-2 expression in the tongue lesions was also suppressed by feeding with garcinol. These results indicate that dietary administration of garcinol inhibited 4-NQO-induced tongue carcinogenesis through suppression of increased cell proliferation activity in the target tissues and/or COX-2 expression in the tongue lesions.

Keywords: Garcinol; Rat; Tongue cancer; 4-Nitroquinoline 1-oxide; Cyclooxygenase-2; Cyclin D1

Abbreviations: BrdU, 5-bromodeoxyurideine; CDK, cyclin dependent kinases; COX, cyclooxygenase; 4-NQO, 4-nitroquinoline 1-oxide; PG, prostaglandin; SCC, squamous cell carcinoma.

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1. Introduction

The incidence and the mortality of oral cancer, which is one of the important public health issues, have increased over the past decades in Europe [1] and in the United States [2]. Although Japan has one of the lowest incidences of oral, lip, and pharyngeal cancers in the world [3], the patients with these malignancies have recently been increasing [4]. Despite recent advances in surgery, chemotherapy, and radiotherapy, the survival of patients with oral carcinoma remains poor [1,2]. Furthermore, patients with oral cancer have an increased incidence of developing second primary tumors of the oral cavity [5,6]. The variation in the incidence of oral cancer in the world is related to exposure to known etiologic agents. It is generally believed that oral carcinomas are caused predominantly by chemical carcinogens, although there is evidence implicating viral, fungal, and physical stimuli in the genesis of some oral neoplasms. Tobacco and alcohol use and the combination of exposure to both are the major risk factors in the development of oral cancer and simultaneous or subsequent second primary cancers [5–10]. One promising approach to reduce the incidence and improve the prognosis of this malignancy is chemoprevention [11]. Dietary factors also play an important role in human health and in the development of certain chronic diseases including cancer [12,13]. Some foods contain antitumor compounds as well as mutagens and/or carcinogens [14]. Such compounds are candidates for chemopreventive agents against cancer development [15].

A polyisoprenylated benzophenone garcinol (Fig. 1, also named camboginol [16] is present in Guttiferae (*Garcinia indica*, *Garcinia huillkensis* and *Garcinia cambogia*). *Garcinia* is a rich source of secondary metabolites including xanthone and flavanoids. In India the dried fruit rind of *G. indica* ('Kokum') is used as a garnish for curry and in some of the folklore medicine and it contains a yellow pigment of garcinol (2–3%, w/w). Garcinol is known to have the same antioxidant property as other chemopreventive agents [17]. We previously reported a possible chemopreventive ability of garcinol in chemically induced colonic preneoplastic lesions in rats [18]. In addition, we demonstrated that garcinol suppresses expression of cyclooxygenase (COX)-2 proteins [18]. Overexpression of COX-2 and elevation of COX-2-mediated prostaglandin (PG) E_2 biosynthesis are involved carcinogenesis in certain organs including oral cavity [19–21].

One of the suitable animal models for field cancerization and for detecting cancer chemopreventive agents is 4-nitroquinoline 1-oxide (4-NQO)-induced rat oral carcinogenesis model [22]. 4-NQO, a water-soluble quinoline derivative, produces a spectrum of preneoplastic and neoplastic lesions in the oral cavity, especially tongue, of rats following 4-NQO application in drinking water. Oral lesions produced by 4-NQO are comparable to human lesions, because many ulcerated and endophytic or exophytic tongue tumors and dysplasia develop when 4-NQO in the drinking water is given to rats or 4-NQO is applied topically to the oral mucosa [23]. Using 4-NQO-induced rat oral carcinogenesis model, we have reported several candidates for chemopreventive agents against oral malignancy [22].

In the current study, possible inhibitory effects of dietary exposure of garcinol during the initiation or post-initiation stages on 4-NQO-induced oral carcinogenesis were investigated in male F344 rats. In addition, the effect of the compound on cell proliferation activity in the tongue was assessed by measuring 5-bromodeoxyuridine (BrdU)-labeling index and cyclin D1-positive cells. The effect of the expression of COX-2 was slso immunohistochemically determined in the tongue lesions induced by 4-NQO.

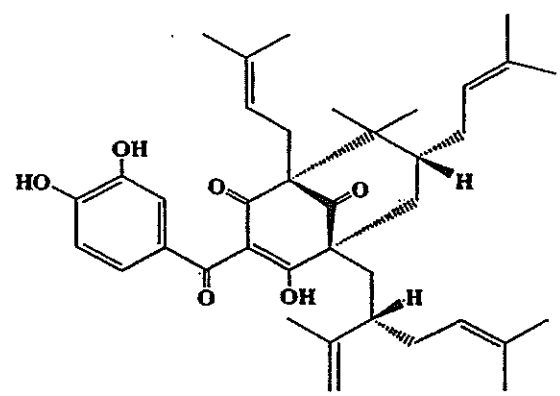

Fig. 1. Chemical structure of garcinol.

2. Materials and methods

2.1. Animals, diets, test chemical, and carcinogen

Male F344 rats, 4 weeks old, were purchased from Charles River Japan (Kanagawa, Japan). After a 2-week quarantine, they were maintained in a holding room under the controlled conditions of a 12 h light/dark cycle, 23±2 °C room temperature, and 50±10% relative humidity. The rats were randomized into experimental and control groups. They were housed three or four to a wire cage. Food and water were available ad libitum. Powdered CE-2 (CLEA Japan, Inc., Tokyo, Japan) was used as a basal diet during the experiment. 4-NQO (Wako Pure Chemical Ind., Osaka, Japan) was given to rats in tap water at a concentration of 20 ppm. Black bottles were used for 4-NQO exposure to protect it from decomposition by light. Garcinol obtained from the Research and Development Division, Kikkoman Co., Ltd was each blended into a powdered basal diet at a dose of 100 or 500 ppm. 4-NQO solution and experimental diets containing garcinol were made on a weekly basis and stored in a dark and cold room (4 °C).

2.2. Experimental procedures

A total of 102 rats were divided into seven groups as shown in Fig. 2 and the tables. At 7 weeks of age, rats in groups 1–5 were given 20 ppm 4-NQO in drinking water for 8 weeks. Groups 2 and 3 were given the diets containing 100 and 500 ppm garcinol, respectively, for 8 weeks, 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 fed the diets mixed with garcinol, at a concentration of 100 or 500 ppm, respectively, starting 1 week after the cessation of 4-NQO treatment, and continued on these diets for 22 weeks. Group 6 was fed the diet containing 500 ppm garcinol alone during the experiment without 4-NQO treatment. Group 7 was given the basal diet and tap water without 4-NQO throughout the experiment and served as an untreated control. All rats were carefully observed daily, and consumption of the drinking water containing 4-NQO and the diets mixed with garcinol was recorded to estimate intake of the chemicals. The experiment was terminated at 32 weeks after the start, and all animals were sacrificed to assess incidences of neoplasms and preneoplastic lesions in all organs, including the oral cavity. Tongues with or without lesions were used for assessing the histopathology and expression of cell proliferation biomarkers by immunohistochemistry. For histological examination, tissue and gross lesions were fixed in 10% buffered formalin, embedded in paraffin blocks, and the histological sections were stained with hematoxylin and eosin (H&E). Epithelial lesions (hyperplasia, dysplasia and neoplasia) in the oral cavity were diagnosed according to the criteria described by Banoczy and Csiba [24] and Kramer et al. [25]. To determine the multiplicity of the tongue lesions, the tongue was examined for gross lesions without the use of any magnification aid. The tongue was cut in half longitudinally and each tissue was fixed in 10% buffered formalin. Each tissue was totally submitted as multiple transverse sections for histological processing. This averaged five to six pieces/tissue and 10–12 pieces/total tongue. The tongue lesions were counted on all slides stained with H&E, the sum was divided by the number of slides, and expressed as mean±SD.

2.3. Determination of proliferative activity in the tongue epithelium

To assess the proliferative activity of the tongue squamous epithelium, the BrdU-labeling indices and cyclin D1-positive cells were quantified. For measurement of BrdU-incorporated nuclei, randomly

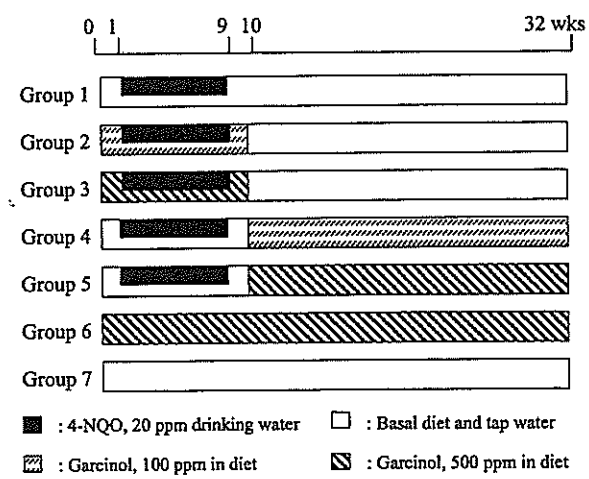

Fig. 2. Experimental protocol.

selected animals (16 rats in group 1, seven rats in group 2, eight rats in group 3, eight rats in group 4, eight rats in group 5, seven rats in group 6, and seven rats in group 7) were given an intraperitoneal injection of 50 mg/kg body weight of BrdU (Sigma Chemical Co., St Louis, MO) 1 h prior to killing. Their tongues were removed and processed to make tissue sections. Paraffin-embedded tongue tissues were cut and four serial sections (3 μm) were made. One section was used for the histopathology, and the others for the immunohistochemistry of BrdU, cyclin D1, and COX-2. For the BrdU-immunohistochemistry, an immunohistochemical analysis kit (DAKO Japan, Kyoto, Japan) was used. The labeling indices of BrdU were calculated by counting at least 474 cells in normal or non-lesional tongue epithelium of each rat. Cyclin D1 immunohistochemistry was done using a stain system kit (DAKO LSAB 2kit/HRP, DAKO Japan Co., Ltd). A mouse monoclonal antibody against cyclin D1 (1:100 dilution, Novocastra Laboratories, Newcastle upon Tyne, UK) was applied to the sections according to the manufacturer's protocol. Slides were subsequently reviewed in a blinded fashion. Cells were considered positive for cyclin D1 when definite nuclear staining was identified. Positive cell ratios for cyclin D1 were calculated by counting at least 513 cells in normal or non-lesional tongue epithelium of each rat.

2.4. COX-2 immunohistochemistry

COX-2 immunohistochemistry was performed using a stain system kit (DAKO). A mouse monoclonal antibody against COX-2 (1:100 dilution, Transduction Laboratories, Lexington, KY) were applied to the sections according to the manufacturer's protocol. Slides were subsequently reviewed in a blinded fashion. Immunohistochemical expression of COX-2 was observed with grading intensity of the immunoreactivity in neoplasms and preneoplastic lesions of tongue. The overall intensity of the staining reaction on each section was scored as follows: ±, no staining or less than 5% of area weakly positive; +, positive (weaker than the staining intensity of macrophages); and ++, strong immunoreactivity (equal or stronger than the staining intensity of macrophages).

2.5. Statistical analysis

Statistical analysis on the incidence of lesions was performed using Yates corrected chi-square test or Fisher's exact probability test, and the data from measurements of body and liver weights, the data from the BrdU labeling index, cyclin D1-positive ratio and COX-2 immunohistochemical stainability were compared by Student's *t*-test or Welch's *t*-test and/or Mann–Whitney *U* test. The results were considered statistically significant if the *P* value was 0.05 or less.

3. Results

3.1. General observations

Animals in groups 1–7 tolerated well the oral administration of 4-NQO and/or garcinol. Mean daily food intakes (16.4 g/rat in group 1, 15.8 g/rat in group 2, 16.5 g/rat in group 3, 16.2 g/rat in group 4, 17.0 g/rat in group 5, 16.6 g/rat in group 6, and 16.8 g/rat in group 7) were insignificant among the groups. Mean daily intake of garcinol/rat was calculated with 1.58 mg in group 2, 8.25 mg in group 3, 1.62 mg in group 4, 8.75 mg in group 5, and 8.30 mg in group 6. There were no significant differences in the total intake of 4-NQO/rat among the five groups (data not shown). The mean body, liver, kidney, and relative liver weights (g/100 g body weight) at the end of the study are indicated in Table 1. The differences in these measures were insignificant among the groups. Dietary administration of garcinol (groups 2–6) did not cause any clinical signs of low survival rate, poor conditions, or histological changes that would point to toxicity in the liver and kidney.

3.2. Incidences and multiplicities of neoplasms and preneoplastic lesions

In this study, neoplasms developed mainly in the dorsal region of the posterior tongue of 4-NQO-treated rats belonging to groups 1–5. Neoplasms were present only in the tongue and no metastasis was noted in any rat. Histopathologically, tongue neoplasms were classified into well-differentiated squamous cell carcinoma (SCC) with or without invasion (Fig. 3a) and squamous cell papilloma. The incidence

Table 1
Body, liver, relative liver, kidney weights in each group

Group no.	Treatment	No. of rats (final)	Body wt (g)	Liver wt (g)	Relative liver wt (g/100 g body wt)	Kidney wt (g)
1	4-NQO alone	26	335.6±16.2[a]	10.8±1.1	3.23±0.37	2.52±0.30
2	4-NQO+100 ppm garcinol	16	324.3±19.4	10.3±1.2	3.18±0.32	2.26±0.51
3	4-NQO+500 ppm garcinol	16	334.4±24.0	11.4±1.0	3.42±0.31	2.63±0.62
4	4-NQO→100 ppm garcinol	15	335.9±17.1	11.1±0.9	3.30±0.26	2.60±0.51
5	4-NQO→500 ppm garcinol	15	328.9±34.0	10.6±1.5	3.23±0.36	2.53±0.52
6	500 ppm garcinol	7	337.9±11.1	10.7±1.0	3.17±0.23	2.71±0.95
7	No treatment	7	330.1±12.6	10.6±0.5	3.20±0.11	2.30±0.37

[a] Mean±SD.

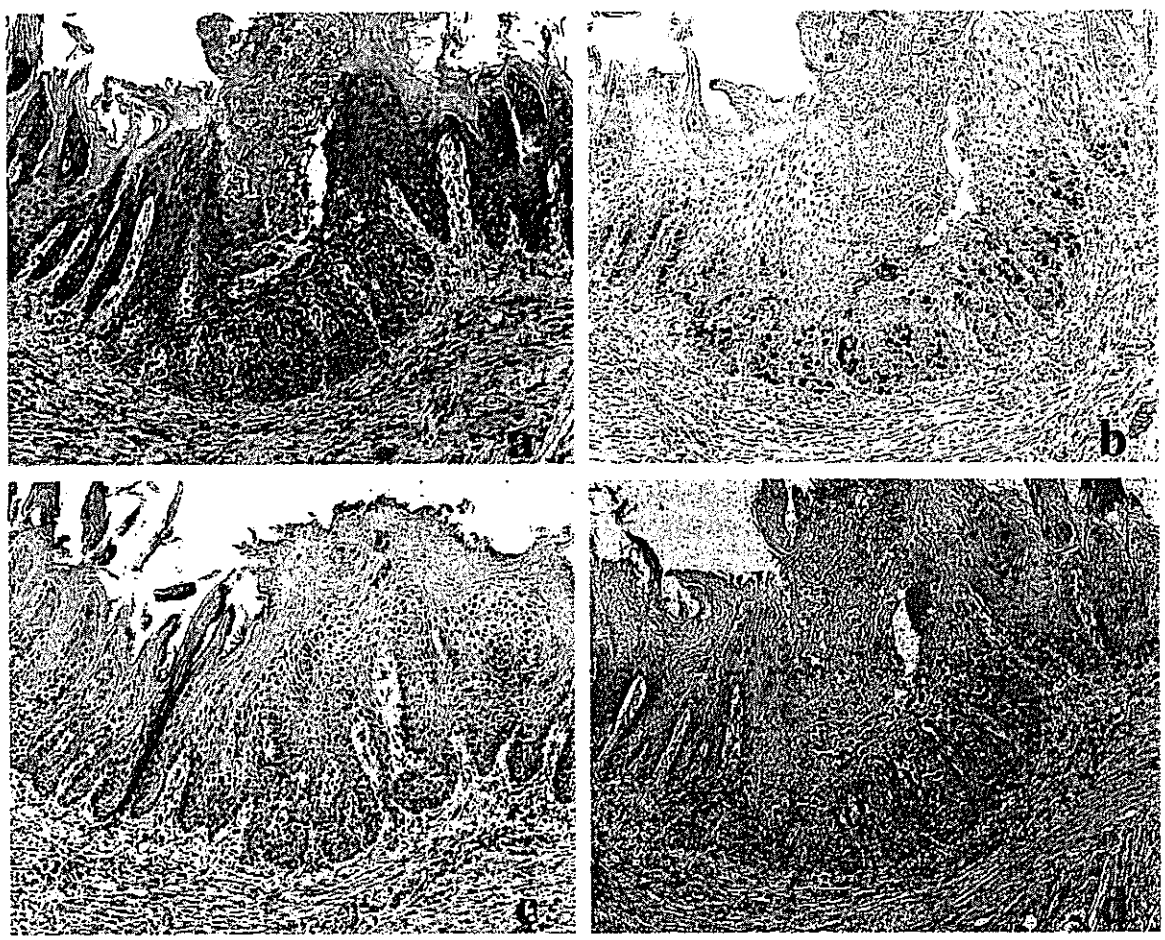

Fig. 3. Histology and immunohistochemistry for COX-2, BrdU, and cyclin D1 of four serial sections from a tongue squamous cell carcinoma in situ developed in a rat of group 1. (a) Squamous cell carcinoma cells replace the entire tongue epithelium. H&E stain. (b) A number of BrdU-positive nuclei are seen in carcinoma cells. BrdU-immunohistochemistry. (c) Cyclin D1-positive nuclei are present in neoplastic cells. Cyclin D1-immunohistochemistry and (d) Most of carcinoma cells express strong positivity of COX-2. COX-2-immunohistochemistry. (a)–(d) Original magnification ×50.

Table 2
Effect of garcinol on development of tongue neoplasms in male F344 rats

Group no.	Treatment	No. of rats	No. of rats with tongue neoplasms			Multiplicity (no. of tumors/rats, mean±SD)	
			Total	Papilloma	SCC	Papilloma	SCC
1	4-NQO alone	26	15 (57.7%)	4 (15.4%)	13 (50.0%)	0.15±0.37	0.50±0.51
2	4-NQO+100 ppm garcinol	16	3 (18.8%)[a]	1 (6.3%)	2 (12.5%)[a]	0.25±0.06	0.13±0.34[b]
3	4-NQO+500 ppm garcinol	16	2 (12.5%)[c]	1 (6.3%)	1 (6.3%)[c]	0.06±0.26	0.07±0.26[d]
4	4-NQO→100 ppm garcinol	15	3 (20.0%)[e]	3 (20.0%)	1 (6.67%)[e]	0.20±0.41	0.07±0.26[d]
5	4-NQO→500 ppm garcinol	15	2 (13.3%)[f]	2 (13.3%)	0 (0%)[g]	0.13±0.35	0
6	500 ppm garcinol	7	0	0	0	0	0
7	No treatment	7	0	0	0	0	0

[a] Significantly different from group 1 by Fisher's exact probability test ($P<0.02$).
[b] Significantly different from group 1 by Student's t-test ($P<0.02$).
[c] Significantly different from group 1 by Fisher's exact probability test ($P<0.005$).
[d] Significantly different from group 1 by Welch's t-test ($P<0.001$).
[e] Significantly different from group 1 by Fisher's exact probability test ($P<0.05$).
[f] Significantly different from group 1 by Fisher's exact probability test ($P<0.01$).
[g] Significantly different from group 1 by Fisher's exact probability test ($P<0.001$).

and multiplicity of neoplasms in each group are given in Table 2. In group 1 (4-NQO alone), the incidence of tongue neoplasms (SCC+papilloma) were 57.7% (15 of 26 rats). On the other hand, only a few rats given garcinol during (groups 2 and 3) or after 4-NQO exposure (groups 4 and 5) had tongue neoplasms: their incidences were 18.8% (three of 16 rats) in group 2, 12.5% (two of 16 rats) in group 3, 20.0% (three of 15 rats) in group 4, and 13.3% (two of 15 rats) in group 5. Statistical analysis revealed a significant decrease in the incidences of tongue neoplasms in groups 2 ($P<0.02$), 3 ($P<0.005$), 4 ($P<0.05$), and 5 ($P<0.01$) when compared with that in group 1. It should be noted that no SCC developed in rats of group 5 (4-NQO→500 ppm garcinol). Also, multiplicities of SCC in groups 2–4 were significantly smaller ($P<0.02$, 0.001, and 0.001, respectively) than that of group 1 (Table 2).

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 1–5, but not in groups 6 and 7. The incidences of such lesions is summarized in Table 3. Tongue squamous cell hyperplasia were

Table 3
Incidence of preneoplastic lesion of tongue in rats of each group

Group no.	Treatment	No. of rats	No. of rats with preneoplastic lesions					
			Total	Hyperplasia		Dysplasia		
				Simple	Papillary	Mild	Moderate	Severe
1	4-NQO alone	26	26 (100%)	13 (50.0%)	22 (84.6%)	7 (26.9%)	12 (46.2%)	17 (65.4%)
2	4-NQO+100 ppm garcinol	16	10 (62.5%)[a]	8 (50.0%)	7 (43.8%)[b]	9 (56.3%)	3 (18.8%)	2 (12.5%)[c]
3	4-NQO+500 ppm garcinol	16	9 (56.3%)[c]	9 (56.3%)	5 (31.3%)[c]	6 (37.5%)	2 (12.5%)[d]	3 (18.8%)[e]
4	4-NQO→100 ppm garcinol	15	7 (46.7%)[c]	7 (46.7%)	5 (33.3%)[a]	4 (26.7%)	4 (26.7%)	2 (13.3%)[a]
5	4-NQO→500 ppm garcinol	15	8 (53.3%)[c]	7 (46.7%)	4 (26.7%)[c]	3 (20.0%)	5 (33.3%)	1 (6.7%)[c]
6	500 ppm garcinol	7	0	0	0	0	0	0
7	No treatment	7	0	0	0	0	0	0

[a] Significantly different from group 1 by Fisher's exact probability test ($P<0.002$).
[b] Significantly different from group 1 by Fisher's exact probability test ($P<0.01$).
[c] Significantly different from group 1 by Fisher's exact probability test ($P<0.001$).
[d] Significantly different from group 1 by Fisher's exact probability test ($P<0.05$).
[e] Significantly different from group 1 by Fisher's exact probability test ($P<0.005$).

classified into two categories of simple and papillary hyperplasia, and tongue squamous dysplasia into three types, mild, moderate, and severe dysplasia according to the degree of cellular atypism. As for squamous cell hyperplasia, the incidence of group 1 was 100% (50.0% with simple hyperplasia and 84.6% with papillary hyperplasia). The incidences of papillary hyperplasia of groups 2 (43.8%), 3 (31.3%), 4 (33.3%), and 5 (26.7%) were significantly lower ($P<0.01$, 0.001, 0.002, and 0.001, respectively) than that (84.6%) of group 1. Also, all rats in group 1 had tongue dysplasia: 26.9% with mild dysplasia, 46.2% with moderate dysplasia, and 65.4% with severe dysplasia). The incidences of severe dysplasia of groups 2 (12.5%), 3 (18.8%), 4 (13.3%) and 5 (6.7%) were significantly lower ($P<0.001$, 0.005, 0.002, and 0.001, respectively) than of group 1 (65.4%). The incidence of moderate dysplasia of group 3 (12.5%) was significantly lower ($P<0.05$) than that of group 1 (46.2%). As shown in Table 4, the multiplicities of papillary hyperplasia of groups 3–5 were significantly smaller ($P<0.005$, 0.001, and 0.001, respectively) than that of group 1. The multiplicities of severe dysplasia of groups 2–5 were significantly smaller ($P<0.001$, 0.005, 0.001, and 0.001, respectively) than that of group 1. Similarly, the multiplicity of moderate dysplasia of group 3 ($P<0.02$) was significantly lower than of group 1. The differences in simple hyperplasia and mild dysplasia were insignificant among the groups.

3.3. BrdU-labeling index and cyclin D1-positive cell rate

The BrdU-labeling indices (%) and cyclin D1-positive cell rates (%) in the non-lesional squamous epithelium are summarized in Table 5. Cells were considered positive for BrdU when definite nuclear staining was identified (Fig. 3b). The BrdU-labeling index of group 1 was the highest among the groups and was significantly larger ($P<0.001$) than that of an untreated control (group 7). Dietary administration of garcinol in groups 2–5 significantly decreased those values ($P<0.005$, 0.001, 0.005, and 0.001, respectively) when compared with group 1. Expression of cyclin D1 in the non-lesional tongue epithelium was defined as positive when definite nuclear staining was identified (Fig. 3c). As indicated in Table 5, the cyclin D1-positive ratio of group 1 was the highest among the groups and was significantly greater ($P<0.001$) than that of an untreated control (group 7). The cyclin D1-positive ratios of groups 2–5 ($P<0.05$, 0.05, 0.001, and 0.001, respectively) were significantly smaller than that of group 1.

3.4. COX-2 expression

Immunoreactivity of COX-2 in the tongue lesions is summarized in Table 6. Slight immunoreactivity for COX-2 was observed in the non-lesional tongue squamous epithelium of rats in groups 1–5. In group 1, COX-2 expression was prominent in the tongue

Table 4
Multiplicity of preneoplastic lesion of tongue in rats of each group

Group no.	Treatment	No. of rats	Multiplicity (no. of preneoplastic lesion/rats, mean ± SD)				
			Hyperplasia		Dysplasia		
			Simple	Papillary	Mild	Moderate	Severe
1	4-NQO alone	26	0.50±0.51	0.85±0.37	0.27±0.45	0.46±0.51	0.65±0.49
2	4-NQO+100 ppm garcinol	16	0.50±0.63	0.44±0.51	0.56±0.73	0.19±0.40	0.13±0.34[a]
3	4-NQO+500 ppm garcinol	16	0.60±0.63	0.33±0.49[b]	0.40±0.51	0.13±0.32[c]	0.20±0.41[d]
4	4-NQO→100 ppm garcinol	15	0.47±0.74	0.33±0.49[a]	0.27±0.46	0.27±0.59	0.13±0.35[a]
5	4-NQO→500 ppm garcinol	15	0.47±0.52	0.27±0.46[a]	0.20±0.41	0.33±0.62	0.07±0.26[e]
6	500 ppm garcinol	7	0	0	0	0	0
7	No treatment	7	0	0	0	0	0

[a] Significantly different from group 1 by Student's t-test ($P<0.001$).
[b] Significantly different from group 1 by Student's t-test ($P<0.005$).
[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 ($P<0.005$).
[e] Significantly different from group 1 by Welch's t-test ($P<0.001$).

Table 5
BrdU-labeling index and cyclin D1 positive ratio on non-lesional area of tongue squamous epithelium

Group no.	Treatment	BrdU-labeling index (%)		Cyclin D1-positive ratio (%)	
		No. of rats examined	Mean±SD	No. of rats examined	Mean±SD
1	4-NQO alone	16	12.96±2.85[a]	26	16.15±2.63[b]
2	4-NQO+100 ppm garcinol	7	9.06±1.58[c]	16	14.08±3.54[d]
3	4-NQO+500 ppm garcinol	8	8.32±1.48[e]	16	13.50±4.25[f]
4	4-NQO→100 ppm garcinol	8	8.99±2.26[c]	15	12.67±3.40[b]
5	4-NQO→500 ppm garcinol	8	7.88±1.30[e]	15	11.03±3.01[b]
6	500 ppm garcinol	7	5.23±0.69	7	8.75±2.49
7	No treatment	7	5.29±0.95	7	8.86±2.41

[a] Significantly different from group 7 by Welch's *t*-test ($P<0.001$).
[b] Significantly different from group 7 by Student's *t*-test ($P<0.001$).
[c] Significantly different from group 1 by Student's *t*-test ($P<0.005$).
[d] Significantly different from group 1 by Student's *t*-test ($P<0.05$).
[e] Significantly different from group 1 by Welch's *t*-test ($P<0.001$).
[f] Significantly different from group 1 by Welch's *t*-test ($P<0.05$).

neoplasms (Fig. 3d) and moderate in hyperplastic or dysplastic lesions, resulting in gradual increase of COX-2 expression during the progressive change in tongue epithelium from hyperplasia or dysplasia to carcinoma. Dietary administration of garcinol in groups 3–5 significantly increased the preneoplastic lesions with negative or weak immunoreactivity when compared to group 1 ($P<0.02$, 0.05, and 0.01, respectively). In addition, dietary administration of 100 ppm garcinol in the post-initiation phase (group 4) significantly decreased COX-2 immunoreactivity in the tongue neoplasms when compared to group 1 ($P<0.02$), although only four neoplasms (three papillomas and one SCC) were examined in group 4. As a result, the immunoreactivity of COX-2 tended to be decreased with

Table 6
COX-2 immunohistochemistry of tongue lesions

Group no.	Treatment	Preneoplastic lesions				Neoplasms			
		No. of lesions examined	No. of lesions with COX-2 antibody staining[a]			No. of lesions examined	No. of lesions with COX-2 antibody staining[a]		
			±	+	++		±	+	++
1	4-NQO alone	36	4 (11.1%)	20 (55.6%)	10 (27.8%)	17	0 (0%)	4 (23.5%)	13 (76.5%)
2	4-NQO+100 ppm garcinol	15	5 (33.3%)	7 (46.7%)	3 (20.0%)	3	1 (33.3%)	1 (33.3%)	1 (33.3%)
3	4-NQO+500 ppm garcinol	14	6 (42.9%)[b,c]	6 (42.9%)	2 (14.3%)	2	0 (0%)	1 (50.0%)	1 (50.0%)
4	4-NQO→100 ppm garcinol	12	5 (41.7%)[d,c]	6 (50.0%)	1 (8.3%)	4	1 (25.0%)	3 (75.0%)	0 (0%)[b]
5	4-NQO→500 ppm garcinol	11	6 (54.5%)[e,c]	4 (36.4%)	1 (9.1%)	2	1 (50.0%)	1 (50.0%)	0 (%)

[a] Staining: ±, negative or less than 5% of area weakly positive; +, positive; and ++, strongly positive.
[b] Significantly different from group 1 by Fisher's exact probability test ($P<0.02$).
[c] Significantly different from group 1 by Mann–Whitney *U*-test ($P<0.05$).
[d] Significantly different from group 1 by Fisher's exact probability test ($P<0.05$).
[e] Significantly different from group 1 by Fisher's exact probability test ($P<0.01$).

dietary exposure to garcinol in both preneoplastic and neoplastic tissues.

4. Discussion

The results in the current study indicated that feeding with garcinol during either the initiation or post-initiation phase effectively suppressed 4-NQO-induced oral carcinogenesis. The inhibition by garcinol might be in a dose-dependent manner. Interestingly no SCC was developed in rats given the higher dose (500 ppm) of garcinol after 4-NQO exposure. The mean intake of 500 ppm garcinol (8.75 mg/day/rat), which caused 100% inhibition of tongue carcioma when fed during the promotion phase of 4-NQO-induced tongue carcinogenesis in rats (group 5), can be estimated as 2.55 g/day in humans (60 kg body weight).

We recently found that dietary exposure of garcinol suppresses the incidence of preneoplastic lesions in the colon of rats initiated with a colonic carcinogen azoxymethane [18]. In the current study, dietary garcinol also suppressed the development of tongue preneoplastic lesions (hyperplasia and dysplasia) induced by 4-NQO. The current results may provide further evidences for the potential of garcinol as a chemopreventive agent in carcinogenesis. In this study, feeding with garcinol-containing diets did not cause retardation of body weight gain and pathological alterations in liver and other organs including kidney, lung, heart, and esophagus. Such results confirm the low toxicity of garcinol observed in our previous study [18].

Inhibitory effect of garcinol on the oral carcinogenesis found in the current study may be caused through several possible mechanisms. One possible mechanism is considered to be the inhibition of cell proliferation activity in the target tissue [26]. Cell proliferation is suggested to play an important role in multistage carcinogenesis [27], including oral tumorigenesis [22,23]. It was reported that most of candidate chemopreventive agents against 4-NQO-induced tongue carcinogenesis can suppress cell proliferation activity in the tongue [22]. As expected, our results showed that feeding with the diets mixed with garcinol suppressed the cell proliferation biomarkers' expression estimated by BrdU-labeling index and cyclin D1-positive cell ratio in the tongue mucosa. Cyclin D1 is a member of the G1 cyclin family, which regulates the transition through a G1 phase of a cell cycle [28,29]. Cyclin D1 in complex with cyclin dependent kinases (CDK4 or CDK6) is an important component of pRb-related tumor suppressor pathway. Cyclin D1/CDK4 or Cyclin D1/CDK6 complexes phosphorylate and inactivate the pRb-protein that leads to a release of E2F-transcription factors needed for the cell cycle progression through the G1-phase [30,31]. Amplification and overexpression of the cyclin D1 gene lead to destabilization of cell cycle control, resulting in uncontrolled cell proliferation. Overexpression of cyclin D1 protein has been reported in human oral cancer and in chemically induced oral carcinoma in rodents [32–34]. In humans, amplification of the cyclin D1 gene was reported to be associated with a decreased survival rate, increased recurrence rate, and more frequent metachronous tumors in the head and neck [35,36]. Thus, our results on cyclin D1 and BrdU immunohistochemistry suggest that the suppressing effects of garcinol on 4-NQO-induced tongue carcinogenesis is partly due to lowered cell proliferation activity caused by feeding of this chemical in the tongue.

In the current study, feeding with garcinol decreased immunohistochemical expression of COX-2 in the tongue preneoplastic and neoplastic lesions. COX enzymes catalyze the conversion of arachidonic acid to PGs and related eicosanoids. COX-2 is inducible by cytokines and growth factors, which play an important role in regulation of inflammation [37]. Recent reports also suggest that up-regulation of COX-2 involves tumor growth and invasiveness through biosynthesis of PGs such as PGE_2. Also, an increased level of PGs and overexpression of COX-2 have been detected in malignant epithelial neoplasms of the head and neck [19,20]. Some non-steroidal anti-inflammatory drugs can inhibit 4-NQO-induced tongue carcinogenesis [38]. Also, selective COX-2 inhibitors have been reported to suppress COX-2 activity, proliferation activity, and PGE_2 production in oral cancer cell lines, and also to inhibit 4-NQO-induced rat tongue carcinogenesis [39–41]. These findings suggest that PGE_2 and/or COX-2 inhibition is a novel target for chemoprevention against oral/tongue cancer. We previously found

that garcinol suppresses COX-2 levels in lipopolysaccharide- and interferon-γ-treated mouse macrophage RAW 264.7 cells [18]. Our findings on COX-2 immunohistochemistry suggest that COX-2 protein was down-regulated in rat tongue lesions by dietary exposure of garcinol, and this may cause inhibition of the progression of tongue carcinogenesis.

In conclusion, the results of our study demonstrate that dietary administration of garcinol isolated from *G. indica* effectively inhibits 4-NQO-induced tongue carcinogenesis in male F344 rats without causing any adverse effects. Together with previous findings [18], garcinol might be a agent that exerts cancer chemopreventive ability in both colon and oral cavity. Further experiments, including preclinical efficacy and mechanistic studies, are warranted to fully evaluate this natural compound for their cancer preventive properties and to understand their mode of action.

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Dose-dependent promoting effect of dextran sodium sulfate on mouse colon carcinogenesis initiated with azoxymethane

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Summary. We previously reported a powerful tumor-promoting ability of dextran sodium sulfate (DSS) in a novel mouse model for colitis-related colon carcinogenesis initiated with azoxymethane (AOM). To determine the dose-dependent influence of DSS in our animal model, male ICR mice were given a single intraperitoneal injection of AOM (10 mg/kg body weight), followed by DSS at dose levels of 2, 1, 0.5, 0.25, and 0.1% (w/v) in drinking water for 1 week. All animals were sacrificed at week 14 and histological alterations in their colon and nitrotyrosine immunohistochemistry were examined to evaluate the nitrosative stress. In the mice which received AOM and 2% DSS, the incidences (multiplicity) of colonic tubular adenoma and adenocarcinoma were 75% (1.25±1.26/mouse) and 100% (2.75±2.22/mouse), respectively. Mice given AOM and 1% DSS had 80% incidence of adenoma (1.00±0.71/mouse) and 60% incidence of adenocarcinoma (1.40±2.07/mouse) in the colon. In a mouse treated with AOM and 0.5% DSS, only one colonic adenoma (20% incidence with 0.20±0.45 multiplicity) developed. Higher frequency of high-grade colonic dysplasia was noted in mice given AOM and 2% or 1% DSS when compared with mice treated with AOM and lower doses of DSS. Also, scoring of inflammation and nitrotyrosine immunoreactivity suggested that severe inflammation and nitrosation stress caused by high-doses (2% and 1%) of DSS contribute its tumor-promoting effects in mouse colon carcinogenesis initiated with a low dose of AOM. Thus, our findings indicate that a tumor-promoting effect of DSS was dose-dependent (1% or more) and the effect might occur under the condition of inflammation and nitrosation stress.

Key words: Dose-dependency, Promotion, DSS, AOM, Mouse colon carcinogenesis

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Introduction

Inflammatory bowel diseases (IBD), including ulcerative colitis (UC) and Crohn's disease (Eaden et al., 2001; van Hogezand et al., 2002) are relatively common in North America, Europe, and Australia. It is well-known that UC patients have a high risk of colorectal cancer (CRC) (Devroede et al., 1971; Kewenter et al., 1978; Greenstein et al., 1979): patients with UC have a 2.0-8.2 relative risk of CRC compared with the normal population, accounting for about 2% of CRC (Hardy et al., 2000).

We recently developed a novel mouse model for colitis-related colon carcinogenesis (Tanaka et al., 2003). In this model, male ICR mice were initiated with a single dose (10 mg/kg body weight) of azoxymethane (AOM) by intraperitoneal (i.p.) injection, and then followed by one-week exposure to 2% dextran sodium sulfate (DSS) in drinking water, starting one week after the injection of AOM. This combined treatment with AOM and DSS resulted in a high incidence and greater multiplicity of colonic neoplasms within 20 weeks. Moreover, the first colonic malignancy was observed as early as 12 weeks into the experimental schedule. These findings suggest a powerful tumor-promoting effect of DSS in our model.

The effects of various tumor-promoters on carcinogenesis are known to be dose-dependent (Pereira et al., 1986) and a lower dose appeared to exhibit a threshold (Maekawa et al., 1992). In colon carcinogenesis, tumor-promoting effect of dietary fat depends on the amount of dietary fat (Reddy and Maeura, 1984). A non-genotoxic carcinogen DSS is widely used for induction of colitis (Okayasu et al., 1990; Cooper et al., 1993), since administration of DSS through diet or drinking water to rodents could induce colonic inflammation which resembled the symptomatic and histopathological findings in humans UC (Okayasu et al., 1990; Cooper et al., 1993). Also, colonic malignancies develop in chronic inflammation induced by long-term administration of DSS, which is similar to

human cases where colorectal adenocarcinoma occurs via the dysplasia-carcinoma sequence (Yamada et al., 1992; Tamaru et al., 1993; Cooper et al., 2000). Severity of mucosal injury caused by DSS relates to the administration dose and duration of DSS (Kitajima et al., 1999; Egger et al., 2000; Shimizu et al., 2003). These findings suggest that the tumor-promoting effect of DSS in our model (Tanaka et al., 2003) is dose-dependent and may be related to mucosal damage by DSS (Tanaka et al., 2001).

In the current study, we investigated the influence of various doses of DSS on our AOM/DSS-induced mouse colon carcinogenesis model to determine the lowest dose of DSS, which can exert its tumor-promoting ability, for utilizing the model for detecting the modifying effects of xenobiotics on colon carcinogenesis. Also, the immunohistochemistry of nitrotyrosine, which is a marker of the formation of peroxynitrite and its interaction with protein tyrosines (Singer et al., 1996), in the colon was performed to determine the possible involvement of inflammation damage by inducible nitric oxide synthase (iNOS), which is induced in inflamed colonic mucosa and is associated with the production of peroxynitrite and nitration of cellular protein in the colon of both human IBD (Singer et al., 1996; Kimura et al., 1998) and chemically-induced colitis of rodents (Zingarelli et al., 1999), in our model.

Materials and methods

Animals, chemicals and diets

Male Crj: CD-1 (ICR) mice (Charles River Japan, Inc., Tokyo), 5 weeks old, were used in this study. They were maintained at Kanazawa Medical University Animal Facility according to the Institutional Animal Care Guidelines. Mice were housed in plastic cages (4 or 5 mice/cage) under controlled conditions of humidity (50±10%), light (12/12 h light/dark cycle), and temperature (23±2 °C). Drinking water and a pelleted basal diet (CRF-1, Oriental Yeast Co., Ltd., Tokyo) were available *ad libitum*. They were quarantined for the first 7 days after arriving, and then randomized by body weight into experimental and control groups. A colonic carcinogen AOM was purchased from Sigma Chemical Co. (St. Louis, MO, USA). DSS with a molecular weight of 40,000 was purchased from ICN Biochemicals, Inc. (Aurora, OH, USA). DSS for induction of colitis was prepared every day by dissolving in distilled water at a concentration of 2, 1, 0.5, 0.25, and 0.1% (w/v).

Experimental procedure

Male ICR mice were divided into 8 groups, as shown in Fig. 1. Mice of groups 1 through 5 were given a single i.p. injection of AOM at a dose of 10 mg/kg body weight. Starting 1 week after the injection, animals of groups 1 through 5 received 2, 1, 0.5, 0.25, and 0.1% DSS in the drinking water for 7 days, respectively, and then were given no further treatment for 12 weeks. Group 6 was given a single i.p. injection of AOM (10 mg/kg body weight) alone. Mice of group 7 received 2% DSS the same as groups 1 through 5. Group 8 was an untreated control. All animals were sacrificed at the end of the study (week 14) by ether overdose. Their large bowel was flushed with saline and excised. Their length (from the ileocecal junction to the anal verge) was measured, cut open longitudinally along the main axis, and then washed with saline. The entire colon was macroscopically inspected, cut, fixed in 10% buffered formalin for at least 24 h, and embedded paraffin for histopathological and immunohistochemical examinations.

Histopathological analysis

Histopathology (mucosal inflammation with or without ulceration, dysplasia, and neoplasms) in the entire colon was analyzed on hematoxylin and eosin-stained sections. Colitis was graded according to the following morphological criteria (Cooper et al., 1993): showing normal appearance (grade 0); shortening and loss of the basal one-third of the actual crypts with mild inflammation in the mucosa (grade 1); loss of the basal two-thirds of the crypts with moderate inflammation in the mucosa (grade 2); loss of the entire crypts with severe inflammation in the mucosa and submucosa, but with retainment of the surface epithelium (grade 3); and presence of mucosal ulcer with severe inflammation (neutrophils, lymphocytes, macrophages, and plasma cells infiltration) in the mucosa, submucosa, muscularis propria, and/or subserosa (grade 4). The scoring was made on the entire colon with or without proliferative lesions and expressed as mean average score / mouse. Colonic mucosa dysplasia (low- and high-grade) and colonic neoplasms were diagnosed according to the earlier reports (Ward, 1974; Riddell et al., 1983; Pascal,

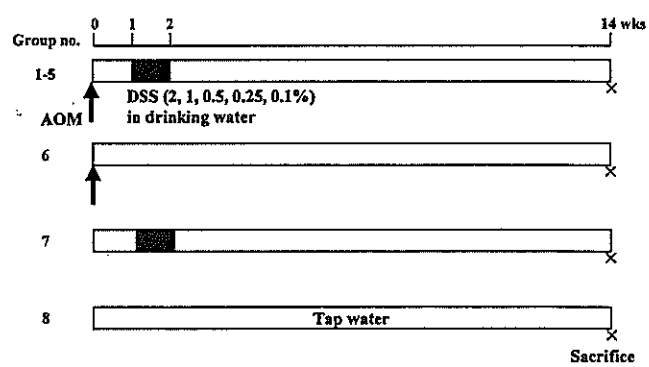

Fig. 1. Experimental protocol. Treatments are as follows: Group 1, AOM (10 mg/kg body weight)→2%DSS; Group 2, AOM→1%DSS; Group 3, AOM→0.5%DSS; Group 4, AOM→0.25%DSS; Group 5, AOM→0.1%DSS; Group 6, AOM alone; Group 7, 2%DSS alone; and Group 8, untreated.

1994). To determine the multiplicity of the colonic mucosal ulcer and dysplasia, the colon was cut into three equal parts from the anus, and then each part was cut in half longitudinally. Each tissue fixed in 10% buffered formation was totally submitted as multiple transverse sections for histological processing. This averaged two pieces/tissue and 12 pieces / total colon. The colon lesions were counted on all slides stained with hematoxylin and eosin, the sum was divided by the number of slides, and expressed as mean ± SD.

Nitrotyrosine immunohistochemistry

Immunohistochemistry was used to evaluate tyrosine nitration, a marker of nitrosative damage in the colon. Paraffin-embedded sections (4 μm) of the colon were deparaffinized, treated with 0.3% hydrogen peroxide for 15 minutes to block endogenous peroxidase activity, and then rinsed briefly in PBS. Non-specific binding was blocked by incubating the slides with a blocking solution (0.1M PBS containing 0.1% triton X-100 and 2% normal goat serum) for 2 hours. Sections were incubated overnight with a primary rabbit polyclonal anti-nitrotyrosine (diluted 1:500, Upstate Biotechnology, Lake Placid, New York, USA) or with control solution. Control sections included buffer alone or non-specific purified rabbit secondary antibody and the avidin-biotin-peroxidase complex (Vectastain Elite ABC kit, Vector Laboratories, Burlingame, CA, USA). Color was developed using 3-3'-diaminobenzidine-4HCl as the chromogen. To quantitate the degree of nitrotyrosine stainability, the grading system (grade 0-4) was used according to the following criteria (Zingarelli et al., 1999): grade 0, no immunoreactivity; grades 1-3, increasing degrees of intermediate immunoreactivity; and grade 4, extensive immunoreactivity. The nitrotyrosine immunohistochemistry was scored on the serial immunostained sections that were made for counting colonic mucosal ulcer and dysplasia.

Statistical analysis

All measurements were compared by the use of Fisher's exact probability test, Student's *t*-test or Welch's *t*-test for paired samples.

Results

General observation

The intake of DSS or tap water did not significantly differ among the groups (data not shown). A few mice receiving AOM and 2% or 1% DSS in the drinking water had bloody stools after the DSS administration. However, no such symptoms were observed in the groups which received AOM and other doses of DSS. Mean body, liver, and relative liver weights (g/100 g body weight) are listed in Table 1. The mean body weight of group 1 (AOM→2% DSS), group 2 (AOM→1% DSS), and group 3 (AOM→0.5% DSS) were significantly lower than that of groups 6 (AOM alone, $P<0.001$ vs. group 1, and $P<0.05$ vs. groups 2 and 3) and 8 (no treatment, $P<0.05$ vs. groups 1 and 2, and $P<0.01$ vs. groups 3). Also, the mean body weight of group 1 was lower ($P<0.05$) than that of group 7 (2% DSS alone). Although the mean liver weight of group 1 was significantly smaller than that of group 6 ($P<0.005$) and group 8 ($P<0.05$), relative liver weights had no statistical differences among the groups. Mean lengths of large bowel of all groups at the end of the study are also given in Table 1. Significant differences were observed between group 1 and group 6 ($P<0.05$) or group 8 ($P<0.01$).

Effects of various doses of DSS on the development of large bowel neoplasms

Macroscopically, flat, nodular, polypoid or caterpillar-like tumors were present in the middle and

Table 1. Body, liver, relative liver weights, and lengths of large bowel in each group.

GROUP no.	TREATMENT (No of mice examined)	BODY WEIGHT (g)	LIVER WEIGHT (g)	RELATIVE LIVER WEIGHT (g/100g body weight)	LENGTH OF LARGE BOWEL (cm)
1	AOM→2%DSS (4)	37.4±5.1[a,b,c,d]	2.27±0.43[d,e]	6.02±0.40	11.8±1.1[f,g]
2	AOM→1%DSS (5)	42.9±2.7[d,f]	2.95±0.16	6.91±0.68	13.7±1.1
3	AOM→0.5%DSS (5)	42.5±2.5[f,g]	2.90±0.23	6.83±0.70	13.7±0.7
4	AOM→0.25%DSS (5)	45.4±3.6	3.02±0.25	6.67±0.64	13.2±1.0
5	AOM→0.1%DSS (4)	48.9±7.0	3.14±0.79	6.37±0.93	13.2±1.3
6	AOM (5)	49.6±4.6	3.14±0.17	6.38±0.55	14.1±1.3
7	2%DSS (5)	45.1±3.4	2.99±0.63	6.60±1.07	13.2±0.8
8	None (5)	47.3±1.5	2.96±0.15	6.27±0.36	13.8±0.5

[a]: mean±SD; [b]: significantly different from group 6 by Student's t-test ($P<0.001$); [c]: significantly different from group 7 by Student's t-test ($P<0.05$): [d]: significantly different from group 8 by Welch's t-test ($P<0.05$); [e]: significantly different from group 6 by Welch's t-test ($P<0.005$). [f]: significantly different from group 6 by Student's t-test ($P<0.05$); [g]: significantly different from group 8 by Student's t-test ($P<0.01$).