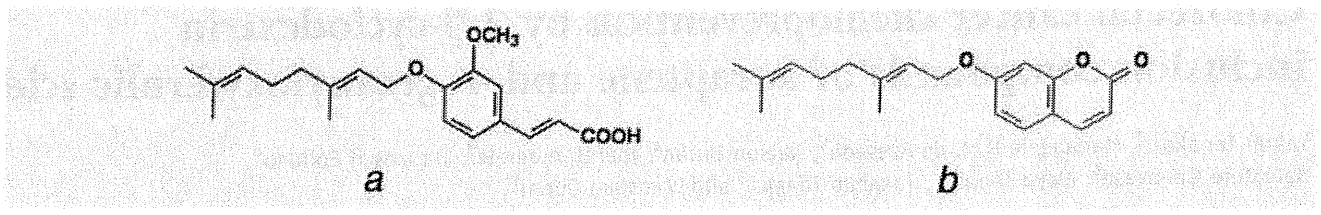

Figure 1. Chemical structures of (*a*) 3-(4′-geranyloxy-3′-methoxyphenyl)-2-*trans* propenoic acid (GOFA) and (*b*) auraptene (AUR).

(IBD), including ulcerative colitis, increases with the increasing extent and duration of the disease.[3,5,6] A mouse model was recently established for colitis-related colon carcinogenesis[7] to facilitate the investigation of pathogenesis[8–10] and the chemoprevention[11,12] of inflammation-related CRC. In this mouse model of inflammation-related two-stage colon carcinogenesis, different types of colonic carcinogens can be used in combination with a colitis-inducing agent, such as dextran sodium sulfate (DSS), and many colonic tumors develop within a short-term period.[7,13–15] The powerful tumor-promoting effect of DSS may be due to the oxidative/nitrosative stress that is caused by DSS-induced colitis.[8–10] This suggests that the oxidative/nitrosative DNA damage associated with inflammation is involved in carcinogenesis, and, therefore, it is important to control the events that result in inflammation-related carcinogenesis.[16] In humans, the inflammatory cytokines and oxidative stress also play a key role in the pathogenesis of IBD-related intestinal damage.[17,18] As our understanding of the pathogenesis of IBD is currently inadequate, drug therapy of IBD and INB-related CRC has been empirical, *i.e.*, it is not based on a sound understanding of the etiology of the disease: drug therapy for IBD initially appears successful in the majority of IBD patients, and it comes with the risk of significant side effects. Therefore, we need new strategies, including chemoprevention, for IBD[19] and IBD-associated CRC.[3,20–23]

The natural and semisynthetic cyclodextrins (CDs) have been extensively studied to improve certain properties of the drugs, such as solubility, stability and bioavailability.[24] The CDs are suitable drug delivery systems because of their ability to greatly modify the physicochemical and biological properties of guest molecules through labile interactions by the formation of inclusion complexes. We have recently shown that modification of the physicochemical properties of violacein was achieved by the preparation of inclusion complexes with β-CD, thus leading to growth-inhibitory effects of its β-CD inclusion complexes against HL60 cells.[25–27] Many drugs currently used in the therapeutic management of colon diseases have been used as inclusion complexes with CDs.[28] The inclusion of the active principles in the cage represented by CD-protected drugs from absorption in the stomach and the upper portion of the lower intestine led to degradation of the saccharide portion in the large bowel by intestinal microflora, thereby ensuring a specific colon delivery with the maximum of bioavailability. This is the scope of the drugs that are being used in the therapy of malignant forms of colon cancer and IBD.

The 3-(4′-geranyloxy-3′-methoxyphenyl)-2-*trans* propenoic acid (4′-geranyloxy-ferulic acid, GOFA) (Fig. 1*a*) is a prenyloxycinnamic acid that was extracted from the Australian small plant *Acronychia baueri* Schott (Family, Rutaceae) in 1966, and, in the last decade, was seen to exert valuable anticancer effects, particularly against tumors affecting the gastrointestinal apparatus.[23,29] Auraptene (AUR) (Fig. 1*b*) is a geranyloxycoumarin that is widespread in the natural kingdom and was extracted from plants belonging to several families (mainly Rutaceae and Apiaceae), comprising many edible fruits and vegetables, such as lemons, grapefruits and oranges. Like GOFA, AUR was seen in recent years to exert valuable pharmacological properties,[30] including dietary feeding colon cancer chemopreventive properties.[22]

As a continuation of our studies, we aimed to acquire further insights into the anticancer properties of selected prenyloxyphenylpropanoids. In our study, we wish to report the colon cancer chemopreventive activity of 2 novel prodrugs of GOFA/β-CD and AUR/β-CD that were obtained as their inclusion complexes with β-CD, using an inflammation-associated mouse colon carcinogenesis initiated with azoxymethane (AOM) and promoted by DSS.[7] For the mechanistic investigation of the effects of the 2 prodrugs on AOM/DSS-induced tumorigenesis, we determined the immunohistochemical expression of the proinflammatory cytokines, including nuclear factor-kappaB (NF-κB),[31–33] NF-E2-related factor 2 (Nrf2),[31,34] tumor necrosis factor (Tnf)-α[35–37] and STAT3[36] in adenocarcinomas that developed in the colon. In addition, the expression of interleukin (IL)-6[38,39] and IL-1β[40] was evaluated in the colonic epithelial malignancies. The effects of GOFA/β-CD and AUR/β-CD in the diet on cell proliferation and apoptosis of colonic adenocarcinomas were evaluated using proliferating cell nuclear antigen (PCNA)[21,41] for proliferative activity, apoptosis indices by terminal deoxynucleotidyl transferase (TdT)-mediated deoxyuridine triphosphate (dUTP)-biotin nick end labeling (TUNEL) method[21] and positive rate of survivin[42] for apoptosis-inhibiting activity.

Material and Methods

Preparation of the inclusion complexes

GOFA and AUR were prepared according to previously reported methods.[43,44] β-CD was purchased from Aldrich Chemical. The inclusion complexes with a 1:1 molar ratio of

GOFA to β-CD and AUR to β-CD (113.5 mg, 0.1 mmol) were obtained by dissolving the geranyloxy derivative (0.1 mmol) in 100 mL of acetone, and soon thereafter, slowly evaporating the solution to dryness under vacuum in a rotatory evaporator at 45°C.[45] The structure of both inclusion compounds was determined by thermal analysis, X-ray diffraction, IR and NMR analysis, as already described.[46] The thermogravimetric analysis (TGA) data were obtained using a Polymer Laboratories (STA-625) thermal analyzer. The samples (2–6 mg) were heated in sealed aluminum pans under nitrogen flow (50 cm^3 min^{-1}) at a heating rate of 10°C min^{-1} from 50 to 500°C.[25,27,45,46] The powder X-ray diffraction patterns were recorded using a 6000-XRD (Shimadzu X-ray diffractometer) under the following conditions: Ni-filtered CuK radiation, voltage 40 kV, current 30 mA, at a scanning speed of 2° min^{-1} and count range 1,000 CPS. The detector was a proportional counter with a 1.7-kV detector voltage.[47–49] The samples of the solid dispersions and the physical mixtures of the complex and free drug and free CD were mixed with KBr and was pressed into a small tablet, which was mounted in the infrared beam. The spectra were recorded on the Perkin Elmer Model 1760X FTIR spectrometer from the KBr discs in the 500–4,000 cm^{-1} region.

Preclinical chemopreventive experiment

Animals, chemicals and diets. Male Crj: CD-1 (ICR) mice (Charles River Japan, Tokyo, Japan), 5 weeks of age, were used in our study. The animals were maintained in the Kanazawa Medical University Animal Facility according to the Institutional Animal Care Guidelines. All the animals were housed in plastic cages (5 mice/cage) and had free access to tap water and a pelleted Charles River Formula (CRF)-1 basal diet (Oriental Yeast, Tokyo, Japan) during quarantine under controlled conditions of humidity (50 ± 10%), lightning (12-hr light/dark cycle) and temperature [(23 ± 2)°C]. They were quarantined for 7 days after arrival and randomized by body weight into the experimental and the control groups. A colonic carcinogen AOM was purchased from Sigma-Aldrich Chemical (St. Louis, MO). DSS with a molecular weight of 36,000–50,000 Da (Lot no. 6046H) was purchased from MP Biomedicals, LLC (Aurora, OH). DSS for the induction of colitis was dissolved in water at 1.5% (w/v). β-CD inclusion complexes of GOFA (GOFA/β-CD) and AUR (AUR/β-CD) were synthesized, as described earlier. The experimental diets containing 0, 100 and 500 ppm of GOFA/β-CD (MW 1465.43) or AUR/β-CD (MW 1433.39) in a powdered basal diet CRF-1 were prepared weekly in our laboratory and stored in a cold room. The doses were selected based on our previous studies.[22,23] The animals had access to food and water at all times. The food cups were replenished daily with a fresh diet. All the handling and procedures were carried out in accordance with the Institutional Animal Care Guidelines.

Experimental procedures. The Institutional Animal Care and Use Committee evaluated all the animal procedures that were associated with our study and assured that all the proposed methods were appropriate.

A total of 150 male ICR mice were divided into 5 experimental and control groups (Supporting Information Fig.). The mice in Groups 1–5 were initiated with AOM by a single intraperitoneal injection (10 mg/kg body weight). One week after the injection, 1.5% DSS (w/v) in drinking water was administrated to mice of Groups 1–5 for 7 days, followed by no further treatment for 18 weeks. The mice of Group 1 were maintained on the CRF-1 diet throughout the study. The mice of Groups 2 and 5 were fed CRF-1 diets containing 100 ppm GOFA/β-CD (Group 2), 500 ppm GOFA/β-CD (Group 3), 100 ppm AUR/β-CD (Group 4) and 500 ppm AUR/β-CD (Group 5) for 15 weeks, respectively, starting 1 week after the cessation of DSS exposure. Group 6 received AOM injection alone. Group 7 was treated with DSS alone. Groups 8 and 9 did not receive AOM and DSS and were fed CRF-1 diets containing 500 ppm GOFA/β-CD and AUR/β-CD, respectively. Group 10 did not receive any treatments and served as an untreated control. At the end of study (Week 18), all the mice were killed by CO_2 asphyxiation for careful necropsy, with emphasis on colon, liver, kidney, lung and heart.

At necropsy, the colons were flushed with saline, excised, their length measured (from ileocecal junction to the anal verge), cut open longitudinally along the main axis and then washed with saline. They were cut and fixed in 10% buffered formalin for at least 24 hr. A histological examination was performed on the paraffin-embedded sections after hematoxylin and eosin (H&E) staining by one (T.T.) of the investigators. Colonic tumors were diagnosed according to Ward's description.[50] In brief, if the tumor cells with tubular formation invaded into the depth of the submucosa, the tumor was diagnosed as adenocarcinoma. When the tumor cells with glandular structure did not invade the submucosa and compressed the surrounding crypts, the tumor was diagnosed as adenoma.

Scoring of inflammation in the large bowel. Inflammation in the large bowel was scored on the H & E-stained sections made from all the mice. For scoring, the large intestinal inflammation was graded according to the following morphological criteria[51]: Grade 0, normal appearance; Grade 1, shortening and loss of the basal 1/3 of the actual crypts with mild inflammation in the mucosa; Grade 2, loss of the basal 2/3 of the crypts with moderate inflammation in the mucosa; Grade 3, loss of the entire crypts with severe inflammation in the mucosa and submucosa, but with retention of the surface epithelium; Grade 4, presence of mucosal ulcer with severe inflammation (infiltration of neutrophils, lymphocytes and plasma cells) in the mucosa, submucosa, muscularis propria and/or subserosa. The scoring was made on the entire colon with or without proliferative lesions and expressed as a mean average score/mouse.

Immunohistochemistry of NF-κB, Nrf2, Tnf-α, Stat3, IL-6, IL-1β, PCNA, TUNEL and survivin. The immunohistochemical analysis of the colon adenocarcinomas for the antibodies of NF-κB, Nrf2, Tnf-α, Stat3, IL-6, IL-1β, PCNA, TUNEL and survivin was performed on 4-μm-thick paraffin-embedded sections by applying the labeled streptavidin biotin method

using a LSAB KIT (DAKO Japan, Kyoto, Japan), with microwave accentuation. The paraffin-embedded sections from the colonic neoplasms of the mice in each group ($n = 18$ in Group 1, $n = 11$ in Group 2, $n = 6$ in Group 3, $n = 6$ in Group 4 and $n = 3$ in Group 5) were heated for 30 min at 65°C, deparaffinized in xylene and rehydrated through graded ethanol at room temperature. Tris-HCl buffer (0.05 M, pH 7.6) was used to prepare the solutions and was used for the washes between the various steps. The incubations were performed in a humidified chamber.

The sections were treated for 40 min at room temperature with 2% bovine serum albumin and incubated overnight at 4°C with primary antibodies. The primary antibodies included anti-NF-κB p50 (H-119) rabbit polyclonal antibody (#sc-7178, 1:500 dilution; Santa Cruz Biotechnology, Santa Cruz, CA), anti-rabbit Nrf2 polyclonal antibody (#ab31163, 1:500 dilution; Abcam, Cambridge, MA), anti-human Tnf-α rabbit polyclonal antibody (#ab6671, 1:500 dilution; Abcam), anti-mouse Stat3 rabbit polyclonal antibody (#ab31370, 1:250 dilution; Abcam), anti-rabbit IL-6 polyclonal antibody (#ab6672, 1:400 dilution; Abcam), anti-mouse IL-1β rabbit polyclonal antibody (#LS-B40, 1:250 dilution; LifeSpan BioSciences, Seattle, WA), anti-rabbit survivin (71G4B7E) monoclonal antibody (#2808, 1:2,000 dilution; Cell Signaling Technology, Danvers, MA) and anti-human PCNA mouse monoclonal antibody (DAKO #U 7032, 1:1,000 dilution; DakoCtytomation, Kyoto, Japan). These antibodies were applied to the sections according to the manufacturer's protocol. The horseradish peroxidase activity was visualized by the treatment with H_2O_2 and 3,3′-diaminobenzidine for 5 min. At the last step, the sections were weakly counterstained with Mayer's hematoxylin (Merck, Tokyo, Japan). For each case, the negative controls were performed on the serial sections without the first antibodies.

The levels of apoptosis in tumor tissues determined by the TUNEL method were done on 4-μm formalin-fixed, paraffin-embedded tissue sections of the colonic adenocarcinomas, according to the manufacturer's instructions using the Apoptosis *in situ* Detection Kit Wako (Cat. No. 298-60201, Wako Pure Chemical Industries, Osaka, Japan). The kit is based on the TUNEL procedure. The appropriate positive and negative controls for determining the specificity of staining were generated. The negative controls were processed in the absence of the TdT enzyme in the reaction buffer. Sections of tissue digested with nuclease enzyme and colon lymphoid nodules, which are known to exhibit high rates of apoptosis, were used as the positive controls. The color was developed with the peroxidase substrate 3,3′-diaminobenzidine and the sections were counterstained with Mayer's hematoxylin (Merck).

Immunohistochemical evaluation and scoring. The immunoreactivity against the antibodies, except PCNA, TUNEL and survivin, was assessed in the large colonic adenocarcinomas (more than 3 mm in diameter) developed in Groups 1–5 using a microscope (Olympus BX41, Olympus Optical, Tokyo, Japan). The intensity and localization of the immunoreactivity against the primary antibodies were determined by a pathologist (T.T.) who was unaware of the treatment group to which the slide belonged. The immunoreactivity was evaluated against the NF-κB, Nrf2, Tnf-α, Stat3, IL-6 and IL-1β antibodies with grading between 0 and 5: 0 (~15% of the colonic cancer cells showing positive reactivity), 1 (16–30% of the colonic cancer cells showing positive reactivity), 2 (31–45% ofthe colonic cancer cells showing positive reactivity), 3 (46–60% of the colonic cancer cells presenting positive reactivity), 4 (61–75% of the colonic cancer cells showing positive reactivity) and 5 (~75% of the colonic cancer cells showing positive reactivity).

The number of nuclei with positive reactivity for PCNA-, TUNEL- and survivin-immunohistochemistry was counted in a total of 3 × 100 cells in 3 different areas of the colonic cancer and expressed as a percentage (mean ± SD).

Statistical evaluation. Where applicable, the data were analyzed using 1-way ANOVA with Tukey-Kramer Multiple Comparisons Test (GraphPad Instat version 3.05, GraphPad Softwear, San Diego, CA) with $p < 0.05$ as the criterion of significance. The Fisher's exact probability test was used for comparison of the incidence of lesions between the 2 groups.

Results

General observation

During the experiment, a few animals of Groups 1–5 and 7 (DSS alone) had bloody stool, but the symptom disappeared soon after stopping the DSS treatment. At Week 18, some of the mice of Groups 1–5 had bloody stool again and anal prolapse because of rectal tumor. The mice belonging to Groups 6 (AOM alone), 8 (GOFA/β-CD alone), 9 (AUR/β-CD alone) and 10 (untreated) did not have any symptoms related to the treatments during the experimental period. As summarized in the Supporting Information Table, there was no significant change between the experimental groups with respect to the parameters tested (body and spleen weights). The liver and relative liver weights of Groups 6 and 8 were significantly smaller in comparison to Group 10. With respect to colon length, the value of Group 1 was significantly lower in comparison to Groups 6 ($p < 0.05$) and 7 ($p < 0.05$). The colon length of Group 3 was significantly larger in comparison to Group 1 ($p < 0.001$).

Pathological findings

Macroscopically, nodular and/or polypoid colonic tumors developed in the middle and distal colon of the mice in Groups 1–5. These tumors were histopathologically tubule adenoma (Fig.2*a*) or adenocarcinoma (well and moderately differentiated) (Fig. 2*b*) with a few adenocarcinomas that invaded into the serosa (Fig. 2*c*). A mucosal ulcer (Fig. 3*a*) was also observed surrounding the neoplasms. The enlarged lymph nodes with inflammation were present around the large bowel with tumors. The mice of Groups 6–10 had no tumors in all the organs examined, including the colon. A

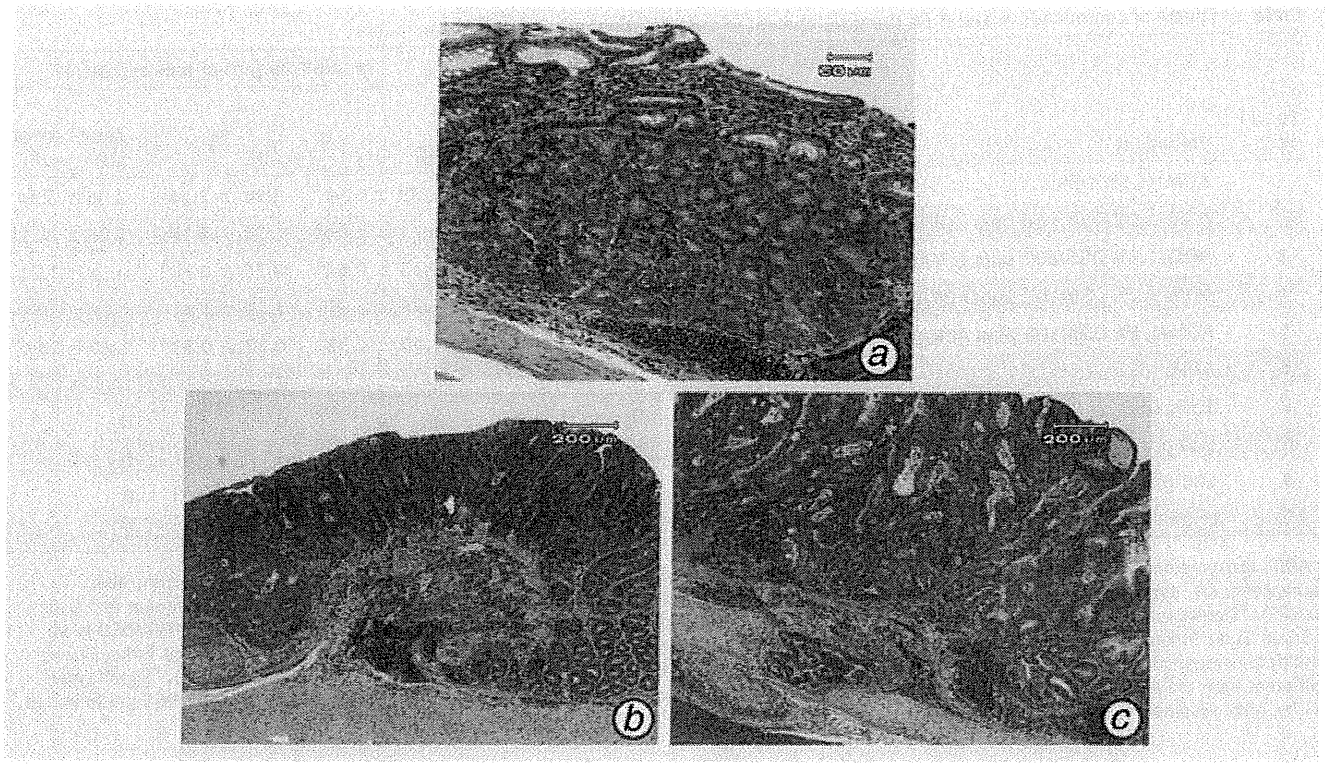

Figure 2. Representative colonic neoplasms induced by azoxymethane (AOM)/dextran sodium sulfate (DSS) in a mouse (Group 1). (*a*) A tubular adenoma, (*b*) a tubular adenocarcinoma with moderately differentiated and (*c*) a tubular adenocarcinoma invaded into the submucosa. Note: The severe inflammation around the tumors; Hematoxylin and eosin stain; the inserted bars indicate magnification (μm).

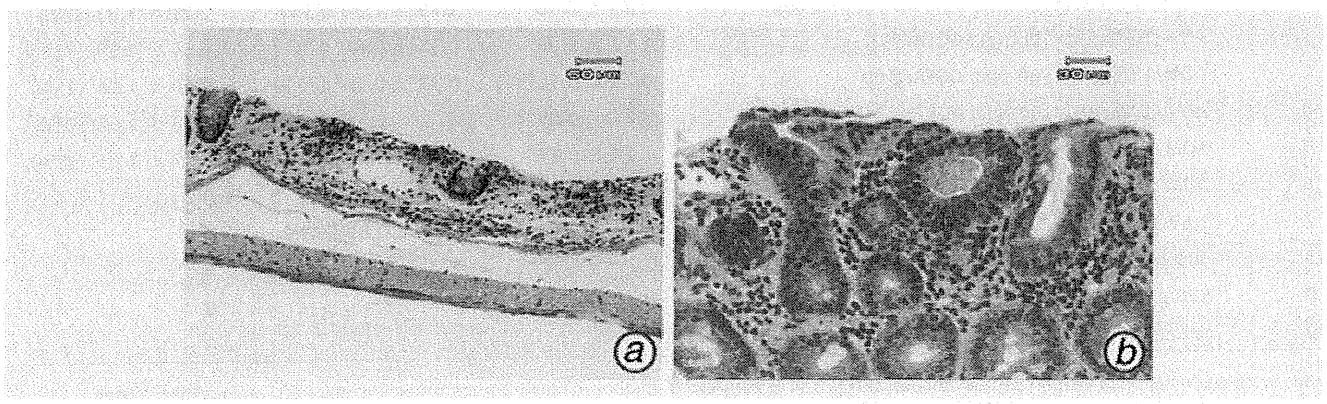

Figure 3. Representative colonic lesions induced by azoxymethane (AOM)/dextran sodium sulfate (DSS) in a mouse (Group 1). (*a*) Mucosal ulcer and (*b*) dysplastic crypts (circled). Hematoxylin and eosin stain, the inserted bars indicate magnification (μm).

mucosal ulcer was observed in the colon of some of the mice of Group 7.

The incidences and multiplicities of the colon neoplasms are summarized in Table 1. Group 1 (AOM/DSS) had 64% incidence of colonic adenocarcinoma with a multiplicity of 1.96 ± 2.24. The incidences of colonic adenocarcinoma of Groups 2 (AOM/DSS → 100 ppm GOFA/β-CD, 24%), 3 (AOM/DSS → 500 ppm GOFA/β-CD, 13%) and 5 (AOM/DSS → 500 ppm AUR/β-CD, 25%) were significantly smaller in comparison to Group 1 ($p < 0.005$, $p = 0.0001$ and $p < 0.005$, respectively). Also, the multiplicities of colonic adenocarcinoma of Groups 2 (0.52 ± 1.16, $p < 0.01$), 3 (0.25 ± 0.74, $p < 0.001$) and 5 (0.42 ± 0.83, $p < 0.05$) were significantly smaller in comparison to Group 1. The incidence (46%) and multiplicity (1.21 ± 1.61) of Group 4 (AOM/DSS → 100 ppm AUR/β-CD) were lower in comparison to Group 1, but the differences between the groups were insignificant. The incidences and multiplicities of colonic adenomas and total colonic tumors in Groups 2–5 were also lower in comparison to Group 1 (Table 1).

Table 1. Effects of compounds A and B on the development of colonic adenoma and adenocarcinoma

Group no.	Treatment	No. of mice examined	Incidence (%)			Multiplicity (no. of tumors/colon)		
			AD	ADC	Total tumors (AD + ADC)	AD	ADC	Total tumors (AD + ADC)
1	AOM[1]/1.5% DSS	28	61	64	71	1.39 ± 1.50[2]	1.96 ± 2.24	3.36 ± 3.34
2	AOM/1.5% DSS/100 ppm GOFA/β-CD	25	40	24[3]	40[4]	0.72 ± 1.06[5]	0.52 ± 1.16[5]	1.24 ± 2.11[6]
3	AOM/1.5% DSS/500 ppm GOFA/β-CD	24	25[7]	13[8]	29[3]	0.33 ± 0.64[6]	0.25 ± 0.74[9]	0.58 ± 1.21[9]
4	AOM/1.5% DSS/100 ppm AUR/β-CD	24	46	46	50	0.96 ± 1.27	1.21 ± 1.61	2.17 ± 2.81
5	AOM/1.5% DSS/500 ppm AUR/β-CD	24	50	25[3]	50	1.00 ± 1.32	0.42 ± 0.83[4]	1.42 ± 2.06[5]
6	AOM	5	0	0	0	0	0	0
7	1.5% DSS	5	0	0	0	0	0	0
8	500 ppm GOFA/β-CD	5	0	0	0	0	0	0
9	500 ppm AUR/β-CD	5	0	0	0	0	0	0
10	Untreated	5	0	0	0	0	0	0

[1]AOM, azoxymethane; DSS, dextran sodium sulfate; GOFA, 3-(4′-geranyloxy-3′-methoxyphenyl)-2-*trans* propenoic acid; CD, cyclodextrin; AUR, auraptene: AD, adenoma; ADC, adenocarcinoma. [2]Mean ± SD. [3]Significantly different from the AOM/DSS group (Group 1) by Chi-square test ($p < 0.005$). [4]Significantly different from the AOM/DSS group (Group 1) by Chi-square test ($p < 0.05$). [5]Significantly different from the AOM/DSS group (Group 1) by Turkey-Kramer multiple comparison post test ($p < 0.01$). [6]Significantly different from the AOM/DSS group (Group 1) by Turkey-Kramer multiple comparison post test ($p < 0.05$). [7]Significantly different from the AOM/DSS group (Group 1) by Chi-square test ($p < 0.01$). [8]Significantly different from the AOM/DSS group (Group 1) by Fisher's exact probability test ($p = 0.0001$). [9]Significantly different from the AOM/DSS group (Group 1) by Turkey-Kramer multiple comparison post test ($p < 0.001$).

Table 2. Effects of compounds A and B on colonic inflammation and development of mucosal ulcer and high-grade dysplasia

Group no.	Treatment	No. of mice examined	Inflammation score (incidence, %)	Number of colonic mucosal ulcer/colon (incidence)	No. of high-grade dysplasia/colon (incidence)
1	AOM[1]/1.5% DSS	28	2.79 ± 0.96[2]	1.29 ± 1.36 (75%)	2.21 ± 1.83 (82%)
2	AOM/1.5% DSS/100 ppm GOFA/β-CD	25	1.52 ± 1.05[3]	0.36 ± 0.64[4] (28%)	0.64 ± 1.19[4] (32%)
3	AOM/1.5% DSS/500 ppm GOFA/β-CD	24	0.75 ± 0.90[3]	0.33 ± 0.56[3] (29%)	0.50 ± 1.14[3] (25%)
4	AOM/1.5% DSS/100 ppm AUR/β-CD	24	1.71 ± 0.69[3]	0.42 ± 0.58[4] (38%)	1.25 ± 1.67 (50%)
5	AOM/1.5% DSS/500 ppm AUR/β-CD	24	1.17 ± 0.87[3]	0.33 ± 0.70[3] (21%)	0.75 ± 1.45[4] (33%)
6	AOM	5	0	0	0
7	1.5% DSS	5	2.20 ± 0.84	2.40 ± 0.89 (80%)	0
8	500 ppm GOFA/β-CD	5	0	0	0
9	500 ppm AUR/β-CD	5	0	0	0
10	Untreated	5	0	0	0

[1]AOM, azoxymethane; DSS, dextran sodium sulfate; GOFA, 3-(4′-geranyloxy-3′-methoxyphenyl)-2-*trans* propenoic acid; CD, cyclodextrin. [2]Mean ± SD. [3]Significantly different from the AOM/DSS group (Group 1) by Turkey-Kramer multiple comparison post test ($p < 0.001$). [4]Significantly different from the AOM/DSS group (Group 1) by Turkey-Kramer multiple comparison post test ($p < 0.01$).

Other colonic lesions, including colitis with or without mucosal ulcer (Fig. 3*a*) and cryptal dysplastia (Fig. 3*b*), were also observed in the colon of mice in Groups 1–5 and/or 7 (Table 2). With respect to the inflammation score (Table 2) determined on H&E-stained sections at Week 18, the value of Group 1 was the highest among the groups and the scores of Groups 2–5 were significantly smaller in comparison to Group 1 ($p < 0.001$ for each comparison). Similarly, as shown in Table 2, the number of colonic mucosal ulcer per colon of Group 1 was the greatest, and the values of Groups 2–5 were significantly smaller in comparison to Group 1 ($p < 0.01$ or $p < 0.001$). The inflammation score and number of mucosal ulcer of Group 7 were the second among the group. Colonic inflammation in the mice of Groups 6, 8, 9 and 10 was slight, if present, and there were mucosal ulcers in the colon of the mice belonging to these groups.

PCNA- and survivin-labeling index in the colonic adenocarcinomas

The data for the PCNA-, TUNEL- and survivin-positive rates of adenocarcinomas are illustrated in Figure 4. As shown in Figure 4*a*, the mean labeling indices of PCNA of Groups 2

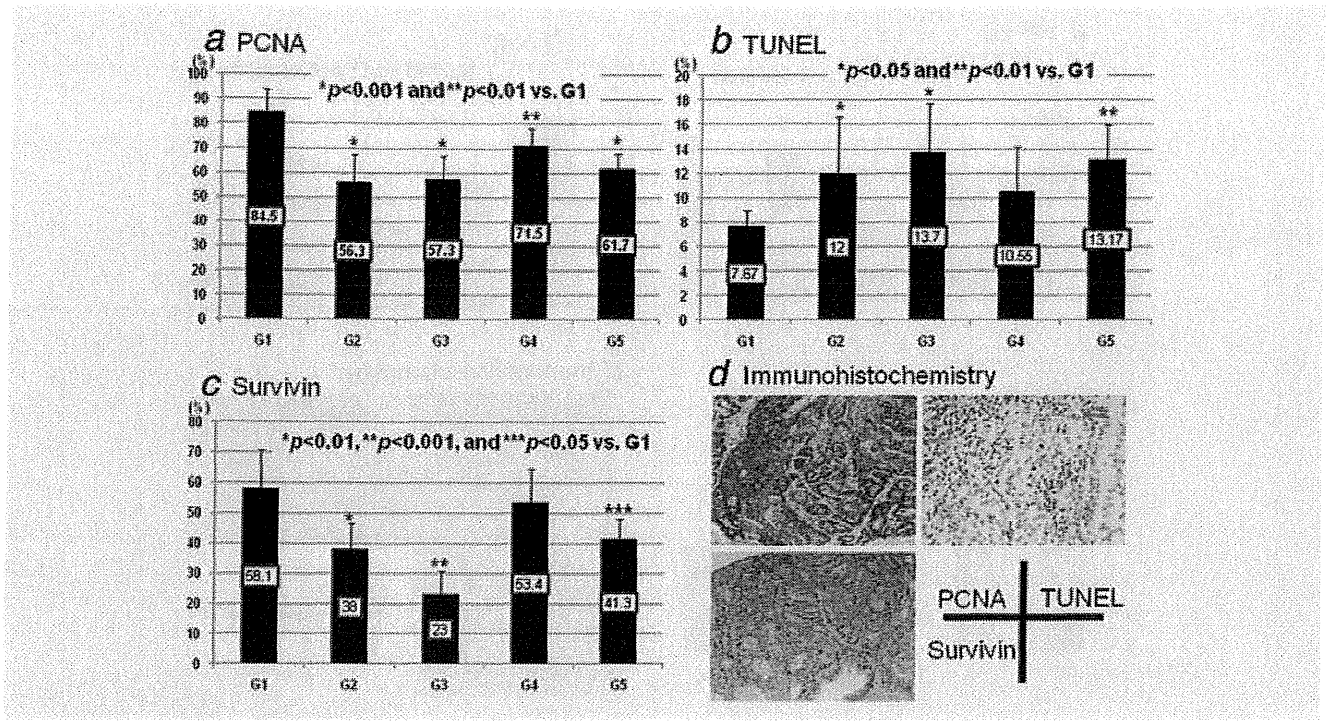

Figure 4. (*a*) The PCNA-labeling index (%, mean ± SD), (*b*) the TUNEL-positive rate (%, mean ± SD) and (*c*) the survivin-positive rate (%, mean ± SD) of colonic adenocarcinomas developed in mice from Groups 1–5. (*d*) The photos show representative PCNA-, TUNEL- and survivin-immunohistochemistry from Group 1.

(56.3 ± 11.2, $p < 0.001$), 3 (57.3 ± 9.6, $p < 0.001$), 4 (71.5 ± 9.4, $p < 0.01$) and 5 (61.7 ± 9.4, $p < 0.001$) were significantly lower in comparison to Group 1 (84.5 ± 9.4) (Fig. 4*d*). The mean TUNEL-positive rates of Groups 2 (12.00 ± 4.56, $p < 0.05$), 3 (13.70 ± 4.04, $p < 0.05$), 4 (10.55 ± 3.62) and 5 (13.17 ± 2.79, $p < 0.01$) were greater in comparison to Group 1 (7.67 ± 1.28) (Fig. 4*d*). With respect to the positive rates of survivin, the values of Groups 2 (38.0 ± 8.5, $p < 0.01$), 3 (23.0 ± 7.6, $p < 0.001$), 4 (53.4 ± 11.3) and 5 (41.3 ± 6.6, $p < 0.05$) were smaller in comparison to Group 1 (58.1 ± 12.6) (Fig. 4*d*).

Scores of NF-κB, Nrf2, Tnf-α, Stat3, IL-6 and IL-1β immunohistochemistry

The data for the scores of the immunohistochemical expression of these proinflammatory cytokines in colonic adenocarcinomas are illustrated in Figures 5*a*–5*c* and 6*a*–6*c*. Adenocarcinomas and the inflammatory mononuclear cells in the colon positively reacted with the antibodies of the proinflammatory cytokines, such as NF-κB, Nrf2, Tnf-α, Stat3, IL-6 and IL-1β (Figs. 5*d* and 6*d*). The scores of NF-κB (Fig. 5*a*), Stat3 (Fig. 6*a*), IL-6 (Fig. 6*b*) and IL-1β (Fig. 6*c*) of Groups 2–5 were significantly lower in comparison to Group 1. Similarly, the mean scores of Nrf2 (Fig. 5*b*) and Tnf-α (Fig. 5*c*) of Groups 2, 3 and 5 were significantly smaller in comparison to Group 1. Both values of Group 3 were lower in comparison to Group 1, but the differences were insignificant.

Discussion

The results of our study clearly indicated that the novel prodrugs, GOFA/β-CD and AUR/β-CD, effectively inhibited AOM/DSS-induced colitis-related colonic carcinogenesis without any adverse effects in mice. The effect of GOFA/β-CD was superior in comparison to AUR/β-CD. Dietary feeding with both prodrugs exerted their cancer chemopreventive ability by modulating cell proliferation, inducing apoptosis and suppressing the proinflammatory cytokines (NF-κB, Nrf2, Tnf-α, Stat3, IL-6 and IL-1β) in adenocarcinomas that developed in the inflamed colon. In turn, the expression of these cytokines may be involved in AOM/DSS-induced colon tumorigenesis. This is the first report showing that prodrugs of GOFA/β-CD and AUR/β-CD exert cancer chemopreventive ability in colitis-related colon carcinogenesis.

In our study, several proinflammatory cytokines were expressed in the colonic tumors and the inflammatory mononuclear cells infiltrated the tumors both internally and peripherally. As the expression of these cytokines may be involved in tumor growth,[52–54] we evaluated the effects of dietary GOFA/β-CD and AUR/β-CD on their expression in adenocarcinomas developed in Groups 1–5. The treatment with GOFA/β-CD and AUR/β-CD significantly lowered colonic inflammation induced by DSS. Chronic inflammation is involved in oncogenesis in certain tissues, including the large bowel. Therefore, the suppression of chronic inflammation through the modulation of expression of several

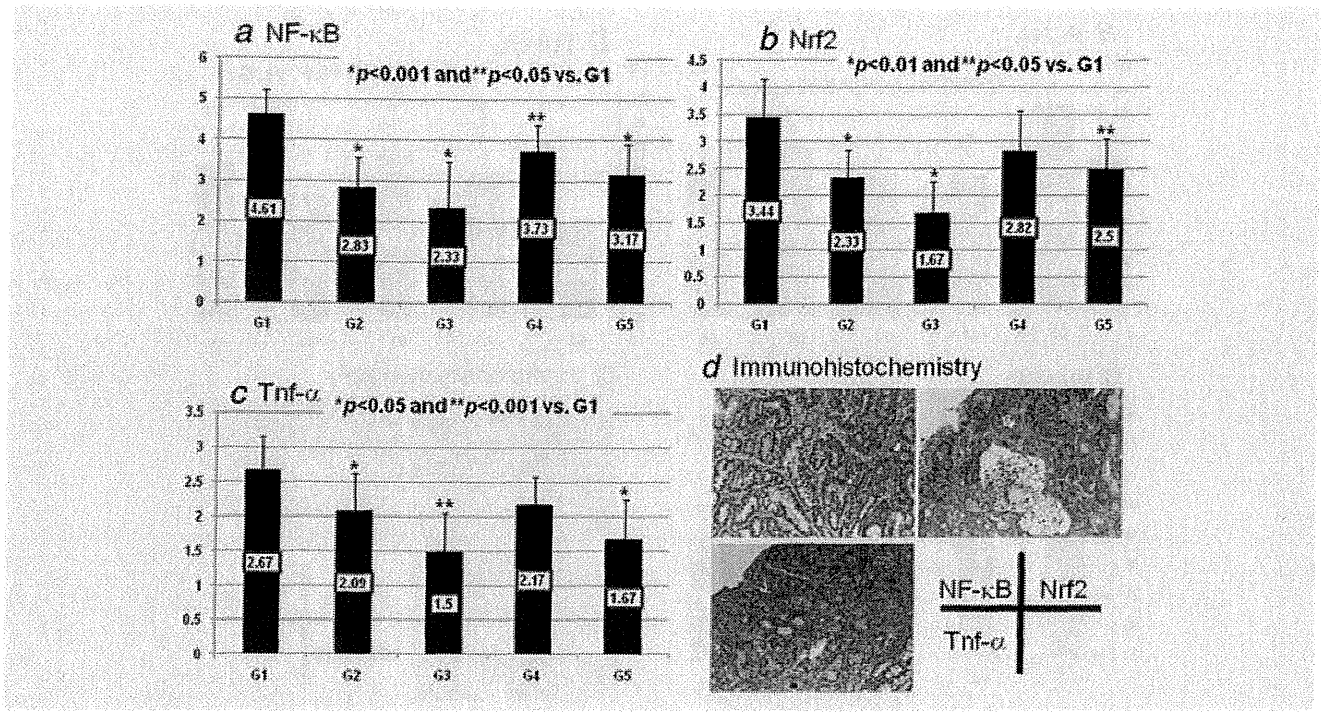

Figure 5. The scores (mean ± SD) of (*a*) NF-κB-, (*b*) Nrf2- and (*c*) Tnf-α-immunoreactivity of colonic adenocarcinomas developed in mice from Groups 1–5. (*d*) The photos show representative NF-κB-, Nrf2- and Tnf-α-immunohistochemistry from Group 1. Note: The adenocarcinoma cells strongly expressed NF-κB, Nrf2 and Tnf-α.

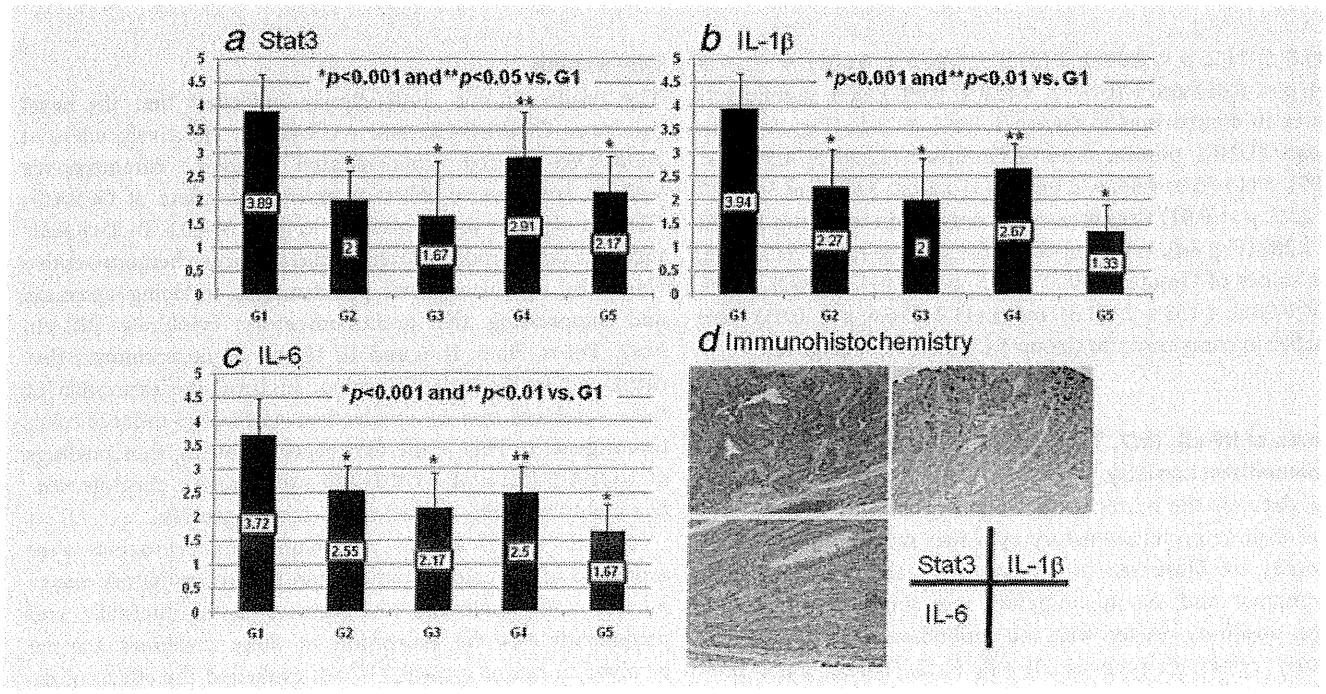

Figure 6. The scores (mean ± SD) of (*a*) Stat3-, (*b*) IL-1β- and (*c*) IL-6-immunoreactivity of colonic adenocarcinomas developed in the mice from Groups 1–5. (*d*) The photos show representative Stat3-, (*b*) IL-1b- and (*c*) IL-6-immunohistochemistry from Group 1. Note: The adenocarcinoma cells strongly expressed Stat3, IL-1β and IL-6.

proinflammatory gene products that mediate several events of carcinogenesis may result in cancer chemoprevention.[55] The modulation of inflammation and expression of cyclooxygenase (COX)-2 and inducible nitric oxide synthase (iNOS) in the colon results in the suppression of colitis-related colon carcinogenesis of mice.[56] Several molecular targets for the

suppression of inflammation-associated carcinogenesis were proposed.[36] In addition to the highly expressed levels of COX-2 and iNOS of colonic adenocarcinomas in our study (data not shown), the proinflammatory cytokines, such as NF-κB, Tnf-α, Stat3, Nrf2, IL-6 and IL-1β, were strongly expressed in adenocarcinomas that developed in the colon of the mice that received AOM and DSS. Moreover, dietary feeding with GOFA/β-CD and AUR/β-CD suppressed their expressions. The Nrf2-deficient mice are susceptible to DSS-induced colitis.[57] IL-6 and IL-1β are involved in the development of IBD and IBD-related colon cancer.[58,59] These proinflammatory cytokines are thus molecular targets for the chemoprevention of inflammation-related carcinogenesis.[31,37,55,60,61] They are candidate biomarkers of colon tumorigenesis,[62,63] because the expression of NF-κB, Tnf-α and IL-1β is involved in colonic tumorigenesis by affecting proliferation and apoptosis.[64–67] The activation of NF-κB, a transcription factor that is activated by several cytokines released during inflammation and is responsible for many of their proinflammatory effects, was shown to promote the growth of the colon tumors in experimental models.[31,55,60,68] Because of the strong link of NF-κB to different stress signals, including cigarette smoke, NF-κB has been called a "smoke-sensor" of the body.[69] In this context, the findings that a tobacco-specific carcinogen enhances AOM/DSS-induced colon carcinogenesis[70] are of interest. In addition, Stat3 expression is an important factor in colon carcinogenesis, tumor invasion[71] and survival/proliferation of the colonic preneoplastic cells.[72] In addition, the anti-inflammatory potential of melatonin through the suppression of the expression of NF-κB and chemokines (IL-8 and monocyte chemoattractant protein) in a rat colitis model[73,74] is of interest, and it is important to further investigate the cancer chemopreventive ability of this bioactive substance, as was done in our study.

In our study, the treatment with both compounds in the diet significantly lowered colonic inflammation induced by DSS. As chronic inflammation involves tumorigenesis and accelerate carcinogenic steps, the suppression of chronic inflammation through the modulation of the expression of several proinflammatory gene products that mediate a critical role in several events of carcinogenesis may result in the inhibition of cancer development, and it may also serve as cancer chemoprevention.[55] AUR and GOFA possess anti-inflammatory activities.[20,75] In addition, we previously reported on the cancer chemopreventive ability of AUR[22,76] and a prodrug, GOFA (called GAP in the study[23]) of the secondary metabolite of ferulic acid in colitis-associated colon carcinogenesis.[23,76] Several molecular targets for the suppression of inflammation-associated carcinogenesis were proposed.[36] Our recent study demonstrated that the modulation of inflammation and the expression of COX-2, iNOS and other proteins in the colon contribute to the suppression of colitis-related colon carcinogenesis.[10,56]

CDs (cyclic oligomers of glucose) that have the properties of forming inclusion complexes with lipophilic drugs have been widely used in therapy to improve water solubility and bioavailability of drugs. Target tissue bioavailability is an important determinant of these efficacies of chemopreventive agents.[77] In our study, we selected β-CD, which is soluble in water and organic solvents. When compared the chemopreventive efficacy of the inclusion complexes of GOFA and AUR with β-CD in our study to that of previous studies,[22,23] GOFA with β-CD was superior to GOFA[23] and AUR with β-CD was less effective in comparison to AUR.[22] This may be related to the differences between the thermal stability of GOFA/β-CD and AUR/β-CD. A thermogram of the AUR/β-CD complex showed that this coumarin derivative melting endotherm had a substantial reduction in peak area, thus implying that the molecular arrangement of AUR in the solid complex was different from the pure crystal compound.[25,27,45,46] Also, the different effects of these compounds with or without b-CD in the activity of matrix metalloproteinases of inflamed colon.[78]

In conclusion, the novel prodrugs of GOFA/β-CD and AUR/β-CD are effective in inhibiting colon cancer development in a two-stage colitis-related mouse colon carcinogenesis through modulation of inflammation, proliferation and the expression of several proinflammatory cytokines (NF-κB, Tnf-α, Stat3, Nrf2, IL-6 and IL-1β) in the inflamed colon of the mice that received AOM and DSS. Our findings therefore support the development of novel site-specifically delivered prodrugs for colon cancer prevention in the inflamed colon.

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Review Article

Understanding Carcinogenesis for Fighting Oral Cancer

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Oral cancer is one of the major global threats to public health. Oral cancer development is a tobacco-related multistep and multifocal process involving field cancerization and carcinogenesis. The rationale for molecular-targeted prevention of oral cancer is promising. Biomarkers of genomic instability, including aneuploidy and allelic imbalance, are able to measure the cancer risk of oral premalignancies. Understanding of the biology of oral carcinogenesis will give us important advances for detecting high-risk patients, monitoring preventive interventions, assessing cancer risk, and pharmacogenomics. In addition, novel chemopreventive agents based on molecular mechanisms and targets against oral cancers will be derived from research using appropriate animal carcinogenesis models. New approaches, such as interventions with molecular-targeted agents and agent combinations in high-risk oral individuals, are undoubtedly needed to reduce the devastating worldwide consequences of oral malignancy.

1. Introduction

Head and neck cancer is the sixth most common human cancer [1], representing 3% of all types of cancer. They are located in the oral cavity in 48% of cases, and 90% of these are oral squamous cell carcinoma [2]. They are sometimes preceded by precancerous lesions, such as leukoplakia and erythroplakia. More than 300,000 new cases worldwide are being diagnosed with oral squamous cell carcinoma annually [3]. Approximately 35,000 new cases are recorded annually in the US [2], 40,000 new cases in the EU, and 10915 new cases in Japan [4]. The most common site for intraoral carcinoma is the tongue, which accounts for around 40% of all cases in the oral cavity proper. Tongue cancers most frequently occur on the posteriorlateral border and ventral surfaces of the tongue. The floor of the mouth is the second most common intraoral location. Less common sites include the gingival, buccal mucosa, labial mucosa, and hard plate.

The incidence of oral cancer has significant local variation. In India and other Asian countries, oral and pharyngeal carcinomas comprise up to half of all malignancies, with this particularly high prevalence being attributed to the influence of carcinogens and region-specific epidemiological factors, especially tobacco and betel quid chewing. An increase in oral cancer prevalence among young adults is a cause of special concern. There has been a 60% increase in the number of under 40 years old with tongue cancer over past 30 years. However, few data have been published on the etiology and natural history of this increase [5]. Oral malignancy including tongue cancer is associated with severe morbidity and less than 50% long-term survival despite advances in treatment (surgery, radiation, and chemotherapy) of oral cancer. The survival of the patients remains very low, mainly due to their high risk of developing a second primary cancer. Thus, early detection and prevention of oral cancer and premalignancy are quite important [6–10]. This paper will focus on our understanding of oral carcinogenesis for preventing and early detection of oral malignancy.

2. Oral Carcinogenesis

Oral carcinogenesis is a highly complex multifocal process that takes place when squamous epithelium is affected by

several genetic alterations. The use of several molecular biology techniques to diagnose oral precancerous lesions and cancer may markedly improve the early detection of alterations that are invisible under the microscope. This would identify patients at a high risk of developing oral cancer [11]. Natural history of oral cancer and sequence of genetic alterations are illustrated in Figure 1. There are approaches to understanding of the molecular basis of oral cancer [12–14]. They include microarray technology, methylation microarrays, gene expression microarrays, array comparative genomic hybridization, proteomics, mitochondrial arrays, and micro-RNA arrays [15]. To date, high-throughout approaches are being used to search for oral cancer biomarkers in biofluids (saliva and serum) [15].

"Field cancerization" refers to the potential development of cancer at multiple sites [16, 17]. This has been observed during the development of cancer in the tissues covered with squamous epithelium (head and neck tumor) and transitional epithelium (urothelial carcinoma). It is evident that oral cancer, like carcinomas in other tissues, develops over many years, and during this period, there are multiple sites of neoplastic transformation occurring throughout the oral cavity. Mutations of this gene have been observed in various sites of premalignant leukoplakia and carcinoma in the same oral cavity [18]. A reduction in tumor suppressor activity by the gene and the development of mutations in *p53* have been associated with smoking and an increased risk for oral carcinoma development [19]. Therefore, multifocal presentations and mutational expressions of tumor suppressor genes may be the consequence of long-term (e.g., 20~40 years) exposure to various environmental and exogenous factors. The continual presence of mutations may also signify changes in DNA repair and apoptosis, thereby increasing the susceptibility for future transformation. Mutational adaptations that modify the survivability of particular clones of transforming cells may also further enhance the level of resistance to therapeutic control. Recent genetic analysis has revealed that cancers developing at distant sites within the oral cavity often are derived from the same initial clone [20]. The multiplicity of the oral carcinogenesis process makes it difficult to interrupt the progression to cancer through surgical removal of a premalignant lesion.

3. Risk Factors of Oral Cancer

The most important risk factor for the development of oral cancer in the Western countries is the consumption of tobacco [21] and alcohol [22]. Although drinking and smoking are independent risk factors, they have a synergistic effect and greatly increase risk together. In Asian countries, the use of smokeless tobacco products such as gutkha and betel quid [5, 23] is responsible for a considerable percentage of oral cancer cases. Several studies have reported a significant familial component in the development of oral cancer. The estimates of risk in the first degree relatives of oral cancer patients vary widely and have been reported to be 1.1 [24] ~ 3.8 [25] although some of these refer to head and neck cancer in general. Familial aggregation of oral cancer, possibly with an autosomal dominant mode of inheritance, was reported in a very small percentage of oral cancer patients [26]. Polymorphic variation of genes in the xenobiotic metabolism pathways, such as in *CYP1A1* or the genes coding for glutathione *S*-transferase-M1 [27, 28] and *N*-acetyltransferase-2 [29] may be implicated. Individuals that carry the fast-metabolizing alcohol dehydrogenase type 3 (*ADH3*) allele [30] may be particularly vulnerable to the effects of chronic alcohol consumption and could be at increased risk to develop oral cancer [31].

Human papilloma virus (HPV), particularly HPV type 16, may be an etiologic factor, especially among persons who do not smoke or drink alcohol [32, 33]. Ang et al. [34] reported that tumor HPV status is a strong and independent prognostic factor for survival among patients with oropharyngeal cancer. They also noted that the risk of death significantly increased with each additional pack year of tobacco smoking. Although the idea that bacterial infections could lead to oral cancer has not been well regarded, there recently has been an increasing body of evidence to suggest a possible relationship between microorganisms and oral cancer development. The most notable example is that of the common pathogenic bacterium *Helicobacter pylori* and its association with gastric cancer. The mouth comprises a variety of different surfaces that are home to a huge diversity of microorganisms, including more than 750 distinct taxa of bacteria, suggesting that the oral squamous epithelium is constantly exposed to a variety of microbial challenges, on both cellular and molecular levels. In this context, we should draw attention to how they may relate to oral cancer development [35, 36].

There are clinically apparent oral premalignant lesions of oral cancer. They include leukoplakia, erythroplakia, nicotine stomatitis and tobacco pouch keratosis, lichen planus, and submucous fibrosis [37]. The term "leukoplakia" first used by Schwimmer in 1877 [38] to describe a white lesion of the tongue probably represented a syphilitic glossitis. The definition of leukoplakia has often been confusing and controversial. Some clinicians now avoid using this term. As defined by the World Health Organization, leukoplakia is "a white patch or plaque that cannot be characterized clinically or pathologically as any other disease [39]". As such, leukoplakia should be used only as a clinical term. The term has no specific histopathological connotation and should never be used as a microscopic diagnosis. In the evaluation of the patient, leukoplakia is a clinical diagnosis of exclusion. Sometimes, a white patch is initially believed to represent leukoplakia, but the biopsy reveals another specific diagnosis. In such cases, the lesion should no longer be categorized as a leukoplakia. Leukoplakia is seen most frequently in middle-aged and older men, with an increasing prevalence with age [40]. Fewer than 1% of men below the age of 30 have leukoplakia, but the prevalence increases to an alarming 8% in men over the age of 70 [40]. The prevalence in women past the age of 70 is approximately 2%. The most common sites are the buccal mucosa, alveolar mucosa, and lower lip. However, lesions in the floor of mouth, lateral tongue, and lower lip are most likely to show dysplastic or malignant changes [41].

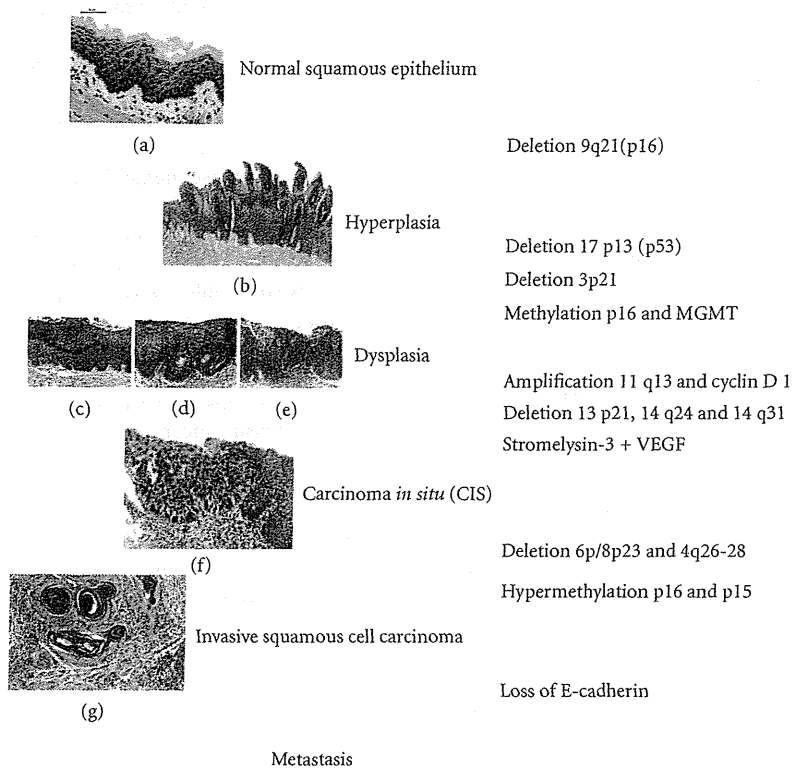

FIGURE 1: Natural history and genetic alterations of oral carcinogenesis. (a), Normal oral mucosa, (b) papillary hyperplasia, (c) midl dysplasia, (d) moderate dysplasia, (e) severe dysplasia, (f) carcinoma in situ, and (g) invasive squamous cell carcinoma (well differentiated).

The term "erythroplasia" originally used by Queyrat [42] to describe a red, precancerous lesion of the penis is used for a clinically and histopathologically similar process that occurs on the oral mucosa. Similar to the definition for leukoplakia, erythroplakia is a clinical term that refers to a red patch that cannot be defined clinically or pathologically as any other condition [39]. This definition excludes inflammatory conditions that may result in a red clinical appearance. Oral erythroplakia occurs most frequently in older men and appears as a red macule or plaque with a soft, velvety texture. The floor of mouth, lateral tongue, retromolar pad, and soft palate are the most common sites of involvement. Often the lesion is well demarcated, but some examples may gradually blend into the surrounding mucosa. Some lesions may be intermixed with white areas (erythroleukoplakia). Erythroplakia is often asymptomatic although some patients may complain of a sore, burning sensation.

Nicotine stomatitis is a thickened, hyperkeratotic alteration of the palatal mucosa that is most frequently related to pipe smoking, but milder examples can also develop secondary to cigar smoking or, rarely, from cigarette smoking [39]. The palatal mucosa becomes thickened and hyperkeratotic, sometimes developing a fissured surface. The surface often develops popular elevations with red centers, which represent the inflamed openings of the minor salivary gland ducts.

Detection and diagnosis of oral neoplasia has traditionally relied heavily on the clinical experience of the examiners and their ability to recognize often subtle morphologic changes. However, some early malignant lesions are clinically indistinguishable from benign lesions, and some patients develop carcinomas in the absence of clinically identifiable oral premalignant lesions. Furthermore, it can be difficult even for experts to determine which oral premalignant lesions are at significant risk to progress to invasive carcinoma. Therefore, an accurate, objective, and noninvasive method to help identify premalignant lesions and to distinguish those at risk of malignant conversion is needed.

4. Biomarkers of Oral Cancer

Biomarkers help in the evaluation of prevention or use of therapies and the detection of the earliest stages of oral mucosal malignant transformation. Biomarkers reveal the genetic and molecular changes related to early, intermediate, and late end points in the process of oral carcinogenesis [43]. These biomarkers will refine our ability to enhance the prognosis, diagnosis, and treatment of oral carcinomas [44]. Genetic and molecular biomarkers will also determine the effectiveness and safety of chemopreventive agents. Chemopreventive agents are chemicals of natural or synthetic

origin. Unlike other drugs, which do not prevent disease, chemopreventive agents reduce the incidence of diseases such as cancer before clinical symptoms occur. This development is critical for the understanding of early oral mucosal transformation. Biomarkers will also reduce the number of patients and the time for long-term follow up required to define a significant clinical response to a chemopreventive agent [45, 46]. The markers may, therefore, clarify the types, doses, frequencies, and regimens to achieve the maximum level of benefit from chemopreventive agents. Decreasing the cost of the clinical trials is another factor that drives the development of biomarkers.

Biomarkers have been categorized following the recommendation by the Committee on Biological Markers of the National Research Council/National Academy of Sciences [47]. They fall into broad groups that detect exposure, progression, susceptibility to carcinogens, and/or the responses by the target cellular populations [46].

A distinct advantage to oral cancer studies is their anatomical access to the developing premalignant and malignant lesions. One could readily analyze biopsies of the primary lesion as well as apparently normal mucosal sites to determine the levels of DNA adducts and oral cancer risk. DNA adduct studies and cytogenetic analyses may also provide evidence for altered structure and function of susceptibility sites in the DNA following DNA-binding studies of nuclear proteins such as p53. Some researchers have focused on microscopic cytogenetic and somatic mutation changes as early biologic markers. One of the markers used to define chromosomal aberrations is the staining for micronuclei in exfoliated buccal mucosal cells [48]. Micronuclei have also been used to evaluate the reversal of leukoplakia and the effectiveness of retinoids, carotenoids, and vitamin E [49, 50]. Other methods include the determination of aneuploidy, and the assessment of losses and gains of genetic material particularly associated with somatic and sex chromosomes. Other sites of chromosomal aberrations are found in sister chromatid exchanges, and allele typic variations designated by losses on chromosomes 3, 4, 5, 6, 8, 9, 11, 13, 17, and 19.

Some molecular biomarkers with potential diagnostic relevance include DNA content and chromosome polysomy, loss of heterozygosity, nucleolar organizer regions, histo-blood group antigens, proliferation markers, increased epidermal growth factor receptor (EGFR), and decreased expression of retinoic acid receptor-β, p16, and p53 [51, 52]. Although a reliable, validated marker panel for providing clinically useful prognostic information in oral premalignant lesions patients has not yet been established, the advent of high throughput genomic and proteomic analysis techniques may soon yield major advances toward a prognostically relevant molecular classification system (Table 1).

Table 1: Potential biomarkers for oral carcinogenesis.

Category	Measures
Genomic biomarker	Micronuclei, DNA adduct, DNA content, and chromosomal aberration (polymorphism, alleic loss, gain, and amplification)
Oncogenic biomarker	Oncogenic expression, modified tumor suppressor genes, and *Src* genes
Proliferation biomarker	Nuclear and cyclin-related antigens, mitotic frequency, ornithine decarboxylase (ODC), and polyamines
Differentiation biomarker	Cytokeratins, transglutaminase Type I, and transcription factor (AP)-1
Oxidative stress biomarker	Glutathione *S*-transferase, stress proteins (HSPs), and Superoxide dismutase
Apoptosis biomarker	Bcl-2 family, chromatin condensation factors, caspases, and nucleosome formation
Immunologic biomarker	Cytokines

5. Animal Models for Oral Carcinogenesis

A variety of animals has been used for the study of tumor growth, the process of carcinogenesis and the prevention/treatment research [8, 53–56]. The continual development of transgenic or knockout mice has improved our understanding of the role of specific genes in tumor growth. The most widely used animal models for oral carcinogenesis are the hamster cheek pouch model [54, 57] and the 4-nitroquinoline 1-oxide- (4-NQO-) induced oral (tongue) carcinogenesis model [8, 53, 58, 59].

In the former model, a complete carcinogen, 7,12-dimethylbenz(*a*)anthracene (DMBA, 0.5%), is applied to the hamster cheek pouch three times a week for 16 weeks. By week 16, all animals exhibit invasive oral squamous cell carcinoma. Many different studies have been conducted with the hamster buccal pouch model, and they have provided an array of changes that are analogous to those observed in human invasive oral carcinoma [54, 57]. These include a mutation in codon 61 of Ha-*ras*, which manifested in an A → T transversion in the second position of codon 61, resulting in an amino acid change from glycine to leucine. The expression of c-Ki-*ras* in malignant tumors of the pouch, but not in the normal oral mucosa, has also been observed at very early stages of tumor development [57]. Although the hamster oral tumor model appears to parallel several changes observed in human oral cancer, the hamster still has several areas of uniqueness which must be considered in any evaluations of results from oral carcinogenesis studies. The hamster cheek pouch provides a relatively large surface area of oral mucosa for the development of invasive carcinoma, while the human does not possess this type of mucosal structure. In contrast to humans, mice, or rats, the hamster cheek pouch lacks lymphatic drainage, which allows various drugs or molecules to accumulate in the pouch. The Syrian hamster population was also derived from a small breeding pair that resulted in a restricted polymorphism for the antigen recognition region (Ia region) and some of the major histocompatibility K and D regions [60]. In addition, the number of T-cells in the hamster spleen exhibits a lower number/gram weight of the organ as compared with the mouse or human [60]. The hamster may also respond to

antigenic tumor sources with a natural killer macrophage or granulocyte cytotoxicity rather than a T cell response [60].

The latter animal models for the study of oral carcinogenesis include those in rats and mice using the water-soluble carcinogen, 4-NQO. The carcinogen is either supplied in the water (20 ppm) for the rats [58, 61–74] or by painting for the mice [75]. Administration with 4-NQO in drinking water (20 ppm) for 8 weeks in rats and mice produces tongue lesions including squamous cell neoplasms (Figure 2) within 32 weeks [71], while topical application of the carcinogen to the mouse palates for up to 16 weeks, just like the hamster model develops palate tumors within 49 weeks [75]. Since the most common site for intraoral carcinoma is the tongue and the drinking water administering of 4-NQO is a simple and easy method, the 4-NQO-induced tongue carcinogenesis model is quite useful for investigating oral carcinogenesis and identifying cancer chemopreventive agents [58, 61–74, 76–84]. In the rat model, with the progression of oral carcinogenesis, increased levels of polyamine synthesis have been noted as well as nucleolar organizing regions (NORs) [58].The mouse model with 4-NQO has demonstrated some molecular mimicry of human oral cancers, as is true of the hamster model [75]. A number of chemical carcinogens including coal tar, 20-methylcholanthrene, DMBA, and 4-NQO have been used in experimental oral carcinogenesis. However, 4-NQO is the preferred carcinogen apart from DMBA in the development of experimental oral carcinogenesis. 4-NQO is a water-soluble carcinogen, which induces tumors predominantly in the oral cavity. It produces all the stages of oral carcinogenesis and several lines of evidences suggest that similar histological as well as molecular changes are observed in the human system. There are several review articles to collate the information available on mechanisms of action of 4-NQO, and studies have been carried out for the development of biomarkers and chemopreventive agents using 4-NQO animal models [8–10, 53, 58, 59, 61–68, 70–74].

The complexity and variety of biochemical changes can increase tumor development is the $p53^{-/-}$ mice [85]. Unfortunately, this model and other genetic mouse models have not been exploited for studying the relationships among chemical oral carcinogenesis, specific genetic defects, and chemoprevention. Genetically altered mouse and rat models have been developed for evaluating molecular-targeted prevention and treatment of oral carcinoma [56]. We have developed *ras*H2 transgenic mouse carcinogenesis model [86] and human c-Ha-*ras* proto-oncogene transgenic rat model [87] for chemoprevention studies on oral (tongue) carcinogenesis.

6. Chemoprevention

Chemoprevention is the use of natural or synthetic substances to halt, delay, or reverse malignant progression in tissues at risk to develop invasive cancer [8–10]. Retinoids are the most extensively studied agents for chemoprevention of oral cancer [88]. 13-*cis*-retinoic acid given for only 3 months produced a clinical response rate of 67% versus 10% for placebo. However, toxicities were considerable, and a very high rate of relapse within 3 months of stopping treatment was reported. Subsequent studies with retinoids in patients with oral premalignant lesions have confirmed clinical and pathologic response rates though toxicities remain a concern [89]. However, translational studies showed that molecular abnormalities persisted in some patients with complete clinical and pathologic response to retinoid therapy [90], suggesting that cancer development may be delayed rather than prevented by these agents. Other agents that have been assessed in clinical trials for chemoprevention activity in oral leukoplakia patients include vitamin E [44], Bowman-Birk inhibitor concentrate (BBIC) derived from soybeans [91], curcumin [92], and green tea polyphenol epigallocatechin-3-gallate. Small clinical trials using oral BBIC revealed no significant toxicity and a 32% response rate [91].

Attention is focused now on the development of agents targeted to specific steps in the molecular progression from normal to oral premalignancy to invasive carcinoma. Examples of molecularly targeted agents that have shown promise in vitro, in animal models, or in early clinical trials include cyclooxygenase (COX)-2 inhibitors and epidermal growth factor receptor EGFR inhibitors [93–95]. Data from several sources suggest that the cyclooxygenase pathway is a good target for oral cancer prevention. COX-2 is overexpressed in head and neck squamous carcinoma [96], and COX-2 inhibitors prevented oral cancer development in animal models [97]. A randomized placebo-controlled trial of the COX-2 inhibitor ketorolac administered as an oral rinse in oral leukoplakia patients revealed that the treatment was well tolerated but did not result in greater clinical response than placebo [98]. However, analysis of the results of this trial are confounded somewhat by the high response rate (32%) in the placebo arm and difficulty in determining whether topical delivery of the agent allowed penetration to the damaged cells. The future of COX-2 inhibitors as chemoprevention agents will also depend on the determination of the extent of risk for cardiac toxicities associated with this class of agents. The EGFR is also a promising molecular target for intervention in oral malignant progression [93–95]. EGFR is a receptor tyrosine kinase that is overexpressed in oral dysplasia and invasive cancer and associated with worse prognosis in patients with head and neck squamous carcinoma [99, 100]. EGFR inhibitors, alone or in combination with chemotherapy and radiotherapy, have shown activity against head and neck squamous carcinoma in clinical trials, and toxicities were generally well tolerated [101]. Evidence has suggested that combination therapy targeting COX-2 and EGFR may be efficacious [95, 102]. Although chemoprevention appears to be a promising approach to managing oral premalignancy, prospective clinical trials using specific agents, and strong corollary translational and laboratory investigations, are needed to evaluate clinical, histologic, and molecular efficacy. In the future, it may be possible and necessary to individualize medical therapy to specific genetic abnormalities detected within the oral mucosa.

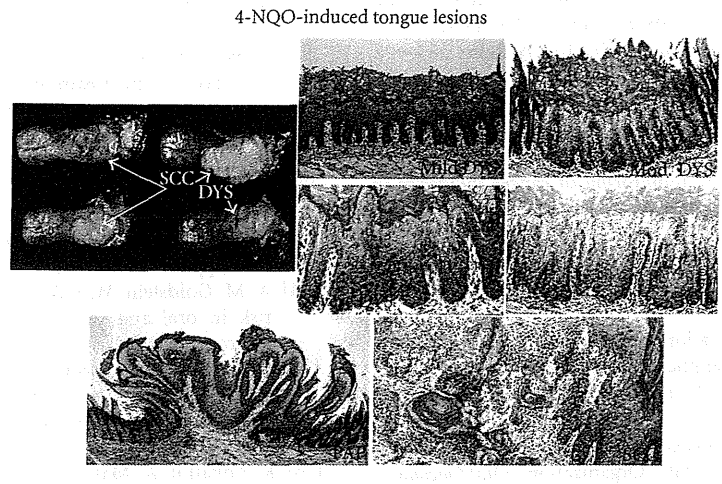

FIGURE 2: 4-NQO-induced tongue lesions in rats. 4-NQO, 4-nitroquinoline 1-oxide; DYS, dysplasia; PAP, papilloma; SCC, squamous cell carcinoma.

7. Conclusion

Human oral cancer being the sixth largest group of malignancies worldwide. Seventy percent of oral cancers appear from premalignant lesions. The process of oral cancer formation results from multiple sites of premalignant change in the oral cavity (field cancerization). Animal models are being widely used, aiming for the development of diagnostic and prognostic markers. The appearance of these premalignant lesions is one distinct feature of human oral cancer. At present, there is dearth of biomarkers to identify which of these lesions will turn into malignancy. Regional lymph node metastasis and locoregional recurrence are the major factors responsible for the limited survival of patients with oral cancer. Paucity of early diagnostic and prognostic markers is one of the contributory factors for higher mortality rates. Determining high- and low-risk populations by measuring reliable biomarkers help us to understand the dynamics and prevention of oral cancer development. The quantitation of genetic and molecular changes and the use of these changes as markers for the detection and prevention of early premalignant change require the harvesting of tissues and cells. Promising technologies are being rapidly developed to assist in localization of abnormal oral mucosa, in noninvasive and objective diagnosis and characterization of identified mucosal lesions, and in therapy of patients with oral cancer. Undoubtedly, the prevention or reduction in the smoking of tobacco products and alcohol consumption would have a profound influence on the incidence of oral cancer. Chemoprevention also has an impact on the development of malignant changes in the oral mucosa. Prevention through chemoprevention and/or the use of systemic medications has been an extensively studied strategy and continues to hold promise as a way of diminishing the morbidity and mortality associated with this malignancy.

Abbreviations

BBIC: Bowman-Birk inhibitor concentrate
COX: Cyclooxygenase
DMBA: 7,12-dimethylbenz(a)anthracene
EGFR: Epidermal growth factor receptor
IL: Interleukin
4-NQO: 4-nitroquinoline 1-oxide.

Conflict of Interests

The authors declared that there is no conflict of interests.

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