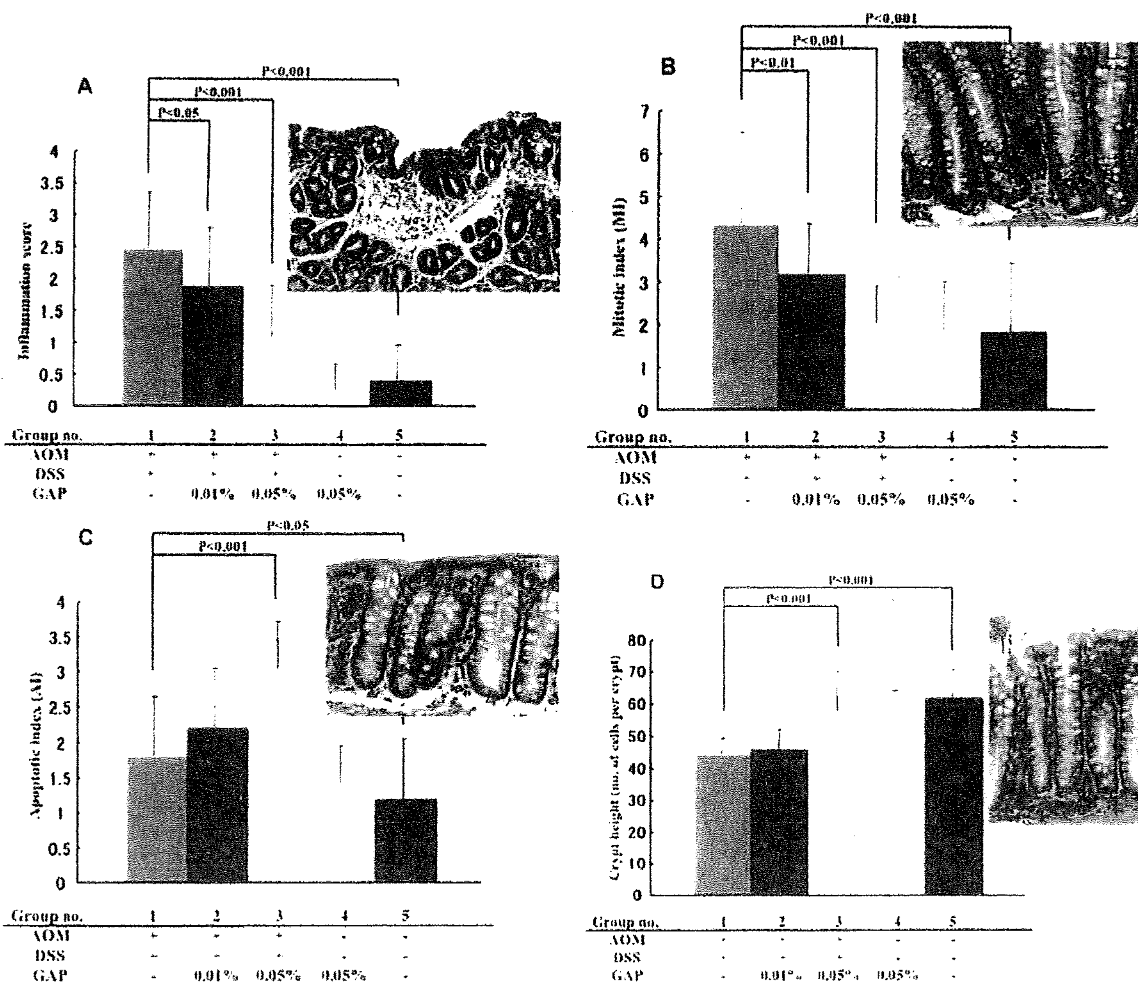

FIG. 3. A: Inflammation score (mean ± SD) of colon from each group. Photo shows inflammation score, Grade 2, in the colon of a mouse from Group 1, hematoxylin and eosin (H & E) stain, a bar inserted indicates magnification (μm). B: Mitotic index (MI, mean ± SD) of crypts of each group. For Fig. 3B photos, arrowheads in green are mitoses, and those in red are apoptotic nuclei or apoptotic bodies, H & E stain; a bar inserted indicates magnification (μm). C: Apoptotic index (AI, mean ± SD) of crypts of each group. For Fig. 3C photo, an arrowhead in green is a mitotic nucleus, and those in red are apoptotic nuclei or apoptotic bodies; H & E stain, a bar inserted indicates magnification (μm). D: Crypt height (number of cells per crypt, mean ± SD) of each group. For the Fig. 3D photo, arrowheads in green are mitoses, and an arrowhead in red is an apoptotic nucleus or apoptotic body. H & E stain; a bar inserted indicates magnification (μm). AOM, azoxymetahen; DSS, dextran sodium sulfate; GAP, 3-(4′-geranyloxy-3′-methoxyphenyl)-2-*trans*-propenoyl-L-alanyl-L-proline

Urinary Level of 8-OHdG

Data on urinary 8-OHdG (ng/mg creatinine) are shown in Fig. 4C. The level of Group 1 (7.10 ± 1.60, $P < 0.001$) was significantly greater than that of Group 5 (3.20 ± 1.79). The values of Groups 2 (4.30 ± 1.57, $P < 0.01$) and 3 (3.70 ± 1.334, $P < 0.001$) were significantly smaller than that of Group 1. The levels of Groups 4 (4.00 ± 1.22) and 5 were comparable.

DISCUSSION

The results of this study clearly indicate that a novel prodrug of the already known colon cancer chemopreventive agent 3-(4′-geranyloxy-3′-methoxyphenyl)-2-*trans*-propenoic acid effectively inhibited AOM/DSS-induced, colitis-related, colonic carcinogenesis without any adverse effects in mice. Dietary feeding with GAP exerted its cancer chemopreventive ability by modulating cell proliferation, suppressing oxidative damage (tissue expression and urinary level of 8-OHdG), and enhancing an antioxidant enzyme, HO-1, in the inflamed colon. This is the first report showing that a prodrug, GAP, exerts cancer chemopreventive ability in colitis-related colon carcinogenesis.

The incidence and multiplicity of colonic tumors in the mice received AOM and 1% DSS in the current study were higher

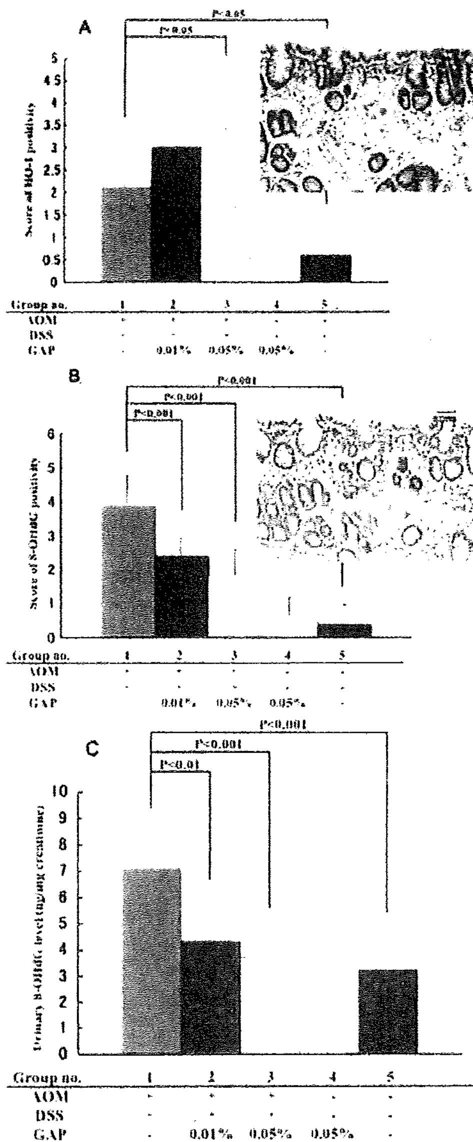

FIG. 4. A: Score (mean ± SD) of heme oxygenase (HO)-1 immunoreactivity. Photo shows strong HO-1 immunoreactivity (Grade 2) of colonic mucosa (same as an inset in Fig. 4) from a mouse of Group 1. Strong positive reaction is present in cryptal cells and inflammatory cells infiltrated into the inflamed colon. HO-1 immunohistochemistry, a bar inserted indicates magnification (μm). B: Score (mean ± SD) of 8-hydroxy-2′-deoxyguanosine (8-OHdG) immunoreactivity. Photo shows strong 8-OHdG immunoreactivity (Grade 3) of colonic mucosa from a mouse of Group 1. Strong positive reaction is present in inflammatory cells in the inflamed colon, and weak reaction is seen in the surface of crypt cells. 8-OHdG immunohistochemistry, a bar inserted indicates magnification (μm). C: Urinary 8-OHdG level (ng/mg creatinine, mean ± SD) of each group. The measurement was done by competitive enzyme-linked immunoabsorbent assay and corrected for urinary creatinine concentration. AOM, azoxymetahen; DSS, dextran sodium sulfate; GAP, 3-(4′-geranyloxy-3′-methoxyphenyl)-2-*trans*-propenoyl-L-alanyl-L-proline.

than our previous dose-response study (40); this may be due to the difference of intake of 1% DSS-containing drinking water: 11.06 ± 0.05 ml/mouse/day in this study and 8.60 ± 0.94 ml/mouse/day in a previous investigation (40). Dietary GAP was able to modulate the endpoints measures in a dose-dependent manner, but the effects on tumor (total tumors and adenoma) multiplicity were comparable. The reason for this is unknown. However, the effects of GAP on the multiplicity of colonic adenocarcinoma suggest the inhibition of progression and the presence of dose-dependent efficacy. Therefore, we should determine the dose-dependency of the inhibition by GAP utilizing 3 or more doses in future studies.

Like ferulic acid (41), our data on 8-OHdG in the colon and urine suggests the antioxidative potential for GAP. Dietary administration of GAP effectively lowered the tissue expression of 8-OHdG in the inflamed colon as well as the urinary level of 8-OHdG. One of the markers of oxidative stress is 8-OHdG, which results from free radical damage to guanine (42). Elevated levels of 8-OHdG have been correlated with malignancy in the colon of rats (43) and humans (44). 8-Oxodeoxyguanosine, the tautomer of 8-OHdG, induces errors in DNA replication, specifically G-to-T transversions (45). Phenolic antioxidants in foods have been shown to reduce markers of oxidative stress and suppress carcinogenesis in certain tissues (46). For example, catechins in tea reduce urinary 8-OHdG content and are effective chemopreventives in the F344 model of colon carcinogenesis (47). In IBD patients, oxidative DNA damage and decrease in antioxidant activity are known (32). We previously reported increased oxidative damage in the inflamed colon of mice treated with DSS (26–28), and modulation of oxidative damage could prevent cancer occurrence (13,29). As found in a phase IIa clinical chemoprevention trial with green tea polyphenols in which urinary 8-OHdG can be monitored to determine oxidative stress condition (48), urinary concentration of 8-OHdG serves as a practical biomarker of oxidative DNA damage in preclinical animal studies.

In the current study, the treatment with GAP in diet significantly lowered colonic inflammation induced by DSS. Because chronic inflammation involves in carcinogenesis, suppression of chronic inflammation through modulation of expression of several pro-inflammatory gene products that mediate a critical role in several events of carcinogenesis may result in cancer chemoprevention (49). Ferulic acid and EGMP have anti-inflammatory effects and inhibition of iNOS expression and thereby suppress carcinogenesis (8,15,23). In fact, our recent study demonstrated that modulation of inflammation and expression of COX-2 and iNOS in the colon contributes to suppression of colitis-related colon carcinogenesis (50). Because several molecular targets for suppression of inflammation-associated carcinogenesis were proposed (51), further studies are warranted for detailed mechanisms by which GAP inhibits inflammation-related carcinogenesis.

Interesting findings observed in this study are that GAP treatment enhanced HO-1 expression in the colon of mice that

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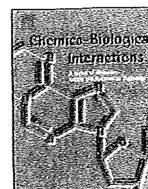

Melatonin suppresses AOM/DSS-induced large bowel oncogenesis in rats

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ABSTRACT

The inhibitory effects of exogenous melatonin (MEL) on colon oncogenesis were investigated using an azoxymethane (AOM)/dextran sodium sulfate (DSS) rat model. Male F344 rats initiated with a single intraperitoneal injection of AOM (20 mg/kg bw) were promoted by 1% (w/v) DSS in drinking water for 7 days. They were then given 0.4, 2 or 10 ppm MEL in drinking water for 17 weeks. At week 20, the development of colonic adenocarcinoma was significantly inhibited by the administration with MEL dose-dependently. MEL exposure modulated the mitotic and apoptotic indices in the colonic adenocarcinomas that developed and lowered the immunohistochemical expression of nuclear factor kappa B, tumor necrosis factor α, interleukin-1β and STAT3 in the epithelial malignancies. These results may indicate the beneficial effects of MEL on colitis-related colon carcinogenesis and a potential application for inhibiting colorectal cancer development in the inflamed colon.

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1. Introduction

The pineal production of melatonin (MEL) is controlled by an endogenous clock, the suprachiasmatic nuclei (SNC) of the hypothalamus, which is synchronized by the light/dark cycle, detected by photoreceptors located at the retinal ganglion cells. Neurons from the SCN project to the superior cervical ganglia and postganglionic noradrenergic fibers innervate pinealocytes. In darkness, the norepinephrine released from these sympathetic fibers promotes the synthesis of MEL. The maximum production of MEL within the pinealocytes occurs at night in response of a signal from the eye indication the absence of light. MEL acts as a circadian rhythm monitor, free radical scavenger and antioxidant, cytoprotective agent, immunomodulator, endocrine modulator, oncostatic agent and thermo regulator [1,2]. A pineal disturbance influences the pathogenesis and the phenotypic variations of metabolic syndrome [3]. Regarding the effects of MEL on oncogenesis, epidemiological studies provided evidence of the potential risk factor of alight at night in breast cancer with its involvement in the entire circadian axis rather than just MEL depression [4], endometrial cancer [5] and colorectal cancer (CRC) [6]. Experimental studies [7] suggest the protective effects of exogenous MEL on carcinogenesis mainly the in mammary gland [8] and other tissues including the colon [9–12], liver [13], skin [14] and pancreas [15]. The protective effects of MEL on oncogenesis are considered to be due to its antioxidative ability [16], antimutagenic potential [17] and alterations of MEL receptor-mediated metabolism [18]. Blask et al. postulated a new mechanism by which physiological and pharmacological blood levels of MEL inhibit cancer growth *in vivo* via a MEL-induced suppression of tumor linoleic acid uptake and its metabolism to the important mitogenic signaling molecule 13-hydroxyoctadecadienoic acid [19]. In addition, MEL is capable of inhibiting chemically induced colitis [20,21]. The anti-inflammatory action of MEL [22] is due to the suppression of COX-2 and iNOS expression [20] and the inhibition of nuclear factor-kappaB (NF-κB) [21]. These findings stimulate the clinical interest of MEL and suggest clinical applications of MEL and MEL agonists in oncology and chemoprevention [1].

Abbreviations: ALT, alanine aminotransferase; AOM, azoxymethane; AST, aspartate aminotransferase; COX, cyclooxygenase; CRC, colorectal cancer; DSS, dextran sodium sulfate; dUTP, deoxyuridine triphosphate; H & E, hematoxylin and eosin; HDL, high-density lipoprotein; IBD, inflammatory bowel disease; IL, interleukin; iNOS, inducible nitric oxide; LDL, low-density lipoprotein; MEL, melatonin; MI, mitotic index; NF-κB, nuclear factor-kappa B; PCNA, proliferative cell nuclear antigen; SNC, suprachiasmatic nuclei; ssDNA, single stranded DNA; T-Cho, total cholesterol; TdT, terminal deoxynucleotidyl transferase; TG, triglycerides; TNF, tumor necrosis factor; TUNEL, TdT-mediated dUTP-biotin nick end labeling; VLDL, very low-density lipoprotein.

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CRC is one of the leading causes of cancer deaths in the Western countries. Globally, the mortality of CRC is 655,000 deaths per year in 2005 [23]. Inflammation is known to be linked with CRC development as it is in epithelial malignancies in other tissues [24]. The risk of CRC in the patients with inflammatory bowel disease (IBD), including ulcerative colitis, increases with the increasing extent and duration of the disease [25]. A mouse model was recently established for colitis-related colon carcinogenesis [26] to investigate the pathogenesis [27–29] and chemoprevention [30,31] of inflammation-related CRC. In this mouse model of inflammation related two-stage colon carcinogenesis, different colonic carcinogens can be used in combination with a colitis-inducing agent, dextran sodium sulfate (DSS) and many colonic tumors develop within a short-term period [26,32–34]. In this model, the powerful tumor promoting effect of DSS may be due to oxidative/nitrosative stress caused by DSS-induced colitis [27–29]. This suggests that oxidative/nitrosative DNA damage associated with inflammation is involved in carcinogenesis and thus it is important to control the events that result in inflammation-related carcinogenesis [35]. In humans, oxidative stress also plays a key role in the pathogenesis of IBD-related intestinal damage [36].

Many drugs and chemopreventive agents are introduced for treatment or chemoprevention of IBD and IBD-related CRC [37]. The current study investigated whether MEL exerts cancer chemopreventive ability in colitis-associated colon carcinogenesis using a rat model [38,39], where the treatment schedule of AOM and DSS was similar to that in the mouse model [26]. In addition, the effects of MEL on the immunohistochemical expression of several biomarkers for colon oncogenesis including NF-κB, tumor necrosis factor (TNF)α, interleukin (IL)-1 and STAT3 [40,41] were studied in the colonic epithelial malignancies (adenocarcinomas). Additionally, effects of MEL on cell proliferation and apoptosis in the colonic adenocarcinomas were evaluated by the proliferation associated indices, proliferative cell nuclear antigen (PCNA) and Ki67 (MIB-5) and the apoptosis indices, the terminal deoxynucleotidyl transferase (TdT)-mediated deoxyuridine triphosphate (dUTP)-biotin nick end labeling (TUNEL) method and the rabbit polyclonal anti-single stranded DNA (ssDNA) method.

2. Materials and methods

2.1. Animals, chemicals and diets

Male F344 rats (Charles River Japan, Tokyo, Japan) aged 5 weeks were used in this study. The animals were maintained in Kanazawa Medical University Animal Facility according to the Institutional Animal Care Guidelines. All animals were housed in plastic cages (3 or 4 rats/cage) with free access to tap water and a pelleted basal diet (CRF-1, Oriental Yeast, Co., Ltd., Tokyo, Japan) under controlled conditions of humidity (50 ± 10%), lightning (12-h light/dark cycle) and temperature (23 ± 2 °C). They were quarantined for 7 days after arrival and randomized by body weight into experimental and control groups. A colonic carcinogen AOM was purchased from Sigma–Aldrich Chemical Co. (St. Louis, MO, USA). DSS with a molecular weight of 36,000–50,000 was obtained from MP Biomedicals, LLC (Aurora, OH). DSS for induction of colitis was dissolved in distilled water at 1% (w/v). MEL (Sigma–Aldrich Chemical Co.) was dissolved in distilled water at concentrations of 0.4, 2 and 10 ppm (w/v) just before used. The preparation was done every day and animals received fresh MEL-containing drinking water in dark bottles. Animals had access to food and drinking water at all times. All handling and procedures were carried out in accordance with the Institutional Animal Care Guidelines.

2.2. Experimental procedures

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

A total of 140 male F344 rats were divided into 9 experimental and control groups, as shown in Table 1. The rats in groups 1 through 6 were initiated by a single intraperitoneal injection of AOM (20 mg/kg body weight). Starting 1 week after the injection, 2% DSS in drinking water was administrated to rats of group 1 ($n = 25$) for 7 days and then followed without any further treatments for 18 weeks. Groups 2–4 ($n = 25$ for each group) were given drinking water containing 0.4, 2 and 10 ppm MEL for 17 weeks, respectively, starting 1 week after the cessation of DSS exposure. Group 5 ($n = 8$) received AOM and 10 ppm MEL. Group 6 ($n = 8$) was given AOM alone. Group 7 ($n = 8$) was given 2% DSS alone. Group 8 ($n = 8$) received 10 ppm MEL alone. Rats of group 9 ($n = 8$) did not receive any treatments and served as an untreated control. MEL was given to rats belonging to groups 2–5, 7 and 8 at night (from 18:00 to 9:00). The highest dose used in this experiment was based on the report by Li et al. [21], in which the dose significantly inhibited colitis-induced 2,4,6-trinitrobenzene by in rats. All animals were subjected to a complete gross necropsy examination at the time of euthanasia by CO_2 asphyxiation (week 20). The body, liver and spleen were weighed.

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 to remove feces. They were cut and fixed in 10% buffered formalin for at least 24 h. The histopathological examination was performed on paraffin-embedded sections, after staining with hematoxylin and eosin (H & E). Colonic tumors were diagnosed according to the Ward's description [42]. In brief, if the tumors with tubular formation invaded into the submucosa, the tumor was diagnosed as an adenocarcinoma. When the tumors with glandular structure did not invade the submucosa or depth and compressed the surrounding crypts, the tumor was diagnosed as an adenoma. The scoring (incidence and multiplicity) of the tumors was done on the H & E-stained tissue sections. The mitotic index (MI) was determined by counting number of mitoses per 100 adenocarcinoma cells on the H & E-stained sections.

2.3. Clinical chemistry

At the end of the 20-week experimental period, 5 rats randomly selected from each group were fasted overnight and then were anesthetized with sodium pentobarbital (30 mg/kg, i.p., Dainippon Pharmaceutical Co., Ltd., Osaka, Japan) for a biochemical analysis. Blood from the inferior vena cava was collected into tubes containing EDTA and centrifuged (1500 × g, 10 min, 4 °C). The serum was aspirated and assayed as described below.

The serum levels of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were assayed using commercially available kits (Wako Pure Chemical Industries, Ltd., Osaka, Japan). Serum total cholesterol (T-Cho), triglycerides (TG), high-density lipoprotein (HDL), low-density lipoprotein (LDL) and very low-density lipoprotein (VLDL) levels were determined using commercial kits (BioVision Incorp., Mountain View, CA, USA). The serum glucose level was measured by the glucose oxidase method (Wako Pure Chemical Industries) and the serum levels of insulin (Wako Pure Chemical Industries) and leptin (GE Healthcare Bio-Sciences Corp., Picscataway, NJ, USA) were determined by sandwich ELISA kits.

Table 1
Effects of melatonin on the development of colonic adenoma and adenocarcinoma.

Group no.	Treatment	No. of rats examined	Incidence (%)		Multiplicity (no. of tumors/colon)	
			AD	ADC	AD	ADC
1	AOM/2% DSS[a]	25	100	100	7.56 ± 1.89[b]	8.16 ± 2.36
2	AOM/2% DSS + 0.4 ppm MEL	25	96	92	5.32 ± 2.19[c]	4.08 ± 2.47[d]
3	AOM/2% DSS + 2 ppm MEL	25	76[e]	72[f]	3.24 ± 2.28[d]	2.36 ± 2.43[d]
4	AOM/2% DSS + 10 ppm MEL	25	68[g]	64[h]	2.28 ± 1.99[d]	1.68 ± 2.08[d]
5	AOM + 10 ppm MEL	8	0	0	0	0
6	AOM	8	0	0	0	0
7	2% DSS	8	0	0	0	0
8	10 ppm MEL	8	0	0	0	0
9	Untreated	8	0	0	0	0

[a] AOM, azoxymethane; DSS, dextran sodium sulfate; MEL, melatonin; AD, adenoma; ADC, adenocarcinoma.
[b] Mean ± S.D.
[c] Significantly different from the AOM/DSS group (group 1) by Turkey–Kramer multiple comparison post-test ($p < 0.01$).
[d] Significantly different from the AOM/DSS group (group 1) by Turkey–Kramer multiple comparison post-test ($p < 0.001$).
[e] Significantly different from the AOM/DSS group (group 1) by Fisher's exact probability test ($p = 0.0111$).
[f] Significantly different from the AOM/DSS group (group 1) by Fisher's exact probability test ($p = 0.0048$).
[g] Significantly different from the AOM/DSS group (group 1) by Fisher's exact probability test ($p = 0.0020$).
[h] Significantly different from the AOM/DSS group (group 1) by Fisher's exact probability test ($p = 0.0008$).

2.4. *Scoring of inflammation in the large bowel*

Two longitudinal H & E-stained sections of a whole colon were made from all rats. Inflammation with or without mucosal ulcer in the large bowel was scored on the H & E-stained sections. For scoring, large intestinal inflammation was graded according to the following morphological criteria [43]: 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 entire crypts with severe inflammation in the mucosa and submucosa, but retaining of the surface epithelium and 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 performed on the entire colon with or without proliferative lesions and expressed as a mean average score/rat.

2.5. *Immunohistochemistry of NF-κB, TNFα, IL-1β, STAT3, PCNA, MIB-5, TUNEL and ssDNA*

The immunohistochemical analysis of the colon adenocarcinoma cells for the NF-κB, TNFα, IL-1β, STAT3, PCNA, MIB-5, TUNEL and ssDNA antibodies was performed on 4-μm-thick paraffin-embedded sections by the labeled streptavidin biotin method using a LSAB KIT (DAKO Japan, Kyoto, Japan), with microwave accentuation. The paraffin-embedded sections 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 washes between the various steps. 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, Inc., Santa Cruz, CA, USA), anti-human TNFα rabbit polyclonal antibody (#ab6671, 1:500 dilution; Abcam, Inc., Cambridge, MA, USA), anti-mouse IL-1β rabbit polyclonal antibody (#LS-B40, 1:250 dilution; LifeSpan BioSciences, Inc., Seattle, WA, USA), anti-mouse STAT3 rabbit polyclonal antibody (#ab31370, 1:250 dilution; Abcam, Inc.), anti-human PCNA mouse monoclonal antibody (DAKO #U 7032, 1:1000 dilution; DakoCtytomation, Kyoto, Japan), mouse monoclonal antibody anti-rat Ki-67 (clone MIB-5, #M7248, 1:100 dilution; DakoCytomation) and anti-ssDNA rabbit polyclonal antibody (#A4506, 1:1000 dilution; DakoCytomation). These antibodies were applied to the sections according to the manufacturer's protocol. Horseradish peroxidase activity was visualized by 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, negative controls were performed on 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 colonic adenocarcinomas, according to manufacturer's instructions using the Apoptosis *in situ* Detection Kit Wako (Cat. No. 298-60201, Wako Pure Chemical Industries, Ltd., Osaka, Japan). The kit is based on the TUNEL procedure. Appropriate positive and negative controls for determining the specificity of staining were generated. 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 positive controls. The color was developed with the peroxidase substrate 3,3′-diaminobenzidine and sections were counterstained with Mayer's hematoxylin (Merck).

2.6. *Immunohistochemical evaluation*

The immunoreactivity against each antibody was assessed in large colonic adenocarcinomas (more than 3 mm in diameter) developed in groups 1–4 using a microscope (Olympus BX41, Olympus Optical Co., Tokyo, Japan). Intensity and localization of immunoreactivity against the primary antibodies were determined by two pathologists (TT and TO) who were unaware of the treatment group to which the slide belonged. They evaluated the immunoreactivity against the NF-κB, TNFα, IL-1β and STAT3 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% of the 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-, MIB-5, ssDNA and TUNEL-immunohistochemistry were counted in a total

of 3×100 cells in three different areas of the colonic cancer and expressed as percentage (mean ± S.D).

2.7. Statistical evaluation

Where applicable, data were analyzed using one-way ANOVA with Tukey–Kramer Multiple Comparisons Test or Bonferroni (GraphPad Instat version 3.05, GraphPad Software, San Diego, CA, USA) 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 two groups.

3. Results

3.1. General observation

During the experiment, a few animals that received AOM/DSS (group 1) or AOM/DSS/MEL (groups 2–4) had bloody stools, but the symptom disappeared soon after stopping of the DSS treatment. At weeks 18–20, some rats of these groups had bloody stools again and anal prolapse with a rectal tumor. Other groups did not show any symptoms that were related to the treatments. The mean daily intakes of DSS (38.5 ± 1.3 ml/day for groups 1–4 and 7), MEL (9.0 ± 1.4 ml/day for groups 2–5 and 8) and distilled water (39.5 ± 0.6 ml/day for groups 6 and 9) were comparable among the groups. The rats of group 8 that received 10 ppm MEL alone for 17 weeks were healthy throughout the study. The mean body weight of group 3 (AOM/DSS/2 ppm MEL, 352 ± 11 g, $p < 0.05$) was greater than that of group 1 (AOM/DSS, 338 ± 12 g) at the end of the study. The mean liver weights did not significantly differ among the groups. The mean relative liver weight (g/100 g body weight) of group 1 (3.25 ± 0.08, $p < 0.001$) was larger than that of the untreated control group (group 9, 2.88 ± 0.05). The values of groups 2 (AOM/DSS/0.4 ppm MEL, 2.87 ± 0.04, $p < 0.001$), 3 (AOM/DSS/2 ppm MEL, 3.13 ± 0.04, $p < 0.001$) and 4 (AOM/DSS/10 ppm MEL, 3.18 ± 0.07, $p < 0.01$) were smaller than that of group 1. However, a histopathological examination revealed no significant morphological alterations in the organs other than the colon.

3.2. Pathological findings

Macroscopically, nodular and polypoid colonic tumors were observed in the middle and distal colon of rats in groups 1 through 4. These tumors were histopathologically tubular adenoma (Fig. 1A) or tubular adenocarcinoma (Fig. 1B), some of which invaded into the submucosa or serosa. Dysplastic crypts (Fig. 1C) and mucosal ulcers (Fig. 1D) were also observed in the surrounding of the neoplasms. Enlarged lymph nodes with inflammation were present around the tumors. Rats of group 5 (AOM/10 ppm MEL), group 6 (AOM alone), group 7 (2% DSS alone), group 8 (10 ppm MEL alone) and group 9 (untreated) had no tumors in all the organs examined, including the large bowel.

3.3. Incidence and multiplicity of colonic tumors and multiplicity of high-grade dysplasia

Table 1 lists the incidences and multiplicities of the colonic tumors. Group 1 (AOM/DSS) had 100% incidence of colon adenocarcinomas with a multiplicity of 8.16 ± 2.36. In contrast, the incidences of colonic adenocarcinoma of group 2 (AOM/DSS/0.4 ppm MEL, 92%), group 3 (AOM/DSS/2 ppm MEL, 72%, $p = 0.0048$) and group 4 (AOM/DSS/10 ppm MEL, 64%, $p = 0.0008$) were low in comparison to that of group 1. Also, the multiplicities of colonic adenocarcinoma of group 2 (4.08 ± 2.47, $p < 0.001$), group 3 (2.36 ± 2.43, $p < 0.001$) and group 4 (1.68 ± 2.08, $p < 0.001$) were significantly lower than that of group 1.

High-grade dysplastic crypts (Fig. 1C) developed in the large bowel of rats in groups 1 through 4 (Table 2). In comparison to group 1 (AOM/DSS), MEL treatment after AOM/DSS exposure significantly lowered the multiplicity of high-grade dysplasia.

3.4. Inflammation score in the colon

Table 2 summarizes data on scores of colonic inflammation at week 20. The mean inflammation score of group 1 (AOM/DSS, 1.88) was the greatest among the groups. The scores of group 2 (AOM/DSS/0.4 ppm MEL, 0.84), group 3 (AOM/DSS/2 ppm MEL, 0.52) and group 4 (AOM/DSS/10 ppm MEL, 0.32) were significantly lower than that of group 1. The value of group 7 (2% DSS) was less than group 1 and larger than groups 2–4. Colonic inflammation was not observed in groups 5 (AOM/10 ppm MEL), 6 (AOM), 8 (10 ppm MEL) and 9 (untreated).

3.5. Indices of mitosis, proliferation and apoptosis in colonic adenocarcinomas

The PCNA-labeling index and MIB-5-positive index of the morphologically intact colonic mucosa ($n = 5$ for each group) were 8.4 ± 1.1 and 9.4 ± 0.5 in group 1, 8.2 ± 0.8 and 8.2 ± 1.5 in group 2, 8.0 ± 0.7 and 7.6 ± 0.9 in group 3, 7.2 ± 0.4 and 7.8 ± 1.3 in group 4, 7.0 ± 0.7 and 7.4 ± 1.3 in group 5, 7.4 ± 0.9 and 7.8 ± 1.8 in group 6, 7.0 ± 1.2 and 7.4 ± 1.7 in group 7, 6.8 ± 0.8 and 7.4 ± 1.1 in group 8 and 6.6 ± 0.9 and 7.2 ± 0.8 in group 9. These values did not show a statistical significant difference among the groups. The data on the proliferative activities in the colonic adenocarcinomas determined by MI, PCNA and MIB-5 indices are illustrated in Fig. 2. As shown in Fig. 2A, the mean MIs of group 2 (AOM/DSS/0.4 ppm MEL, 25.8), group 3 (AOM/DSS/2 ppm MEL, 22.7) and group 4 (AOM/DSS/10 ppm MEL, 18.1) were smaller than group 1 (AOM/DSS, 27.3). The PCNA-labeling indices (Fig. 2B) of group 2 (AOM/DSS/0.4 ppm MEL, 65.0), group 3 (AOM/DSS/2 ppm MEL, 54.6) and group 4 (AOM/DSS/10 ppm MEL, 49.0) were significantly smaller than that of group 1 (AOM/DSS, 72.8). In addition, the MIB-5-labeling indices (Fig. 2C) of group 2 (AOM/DSS/0.4 ppm MEL, 70.3), group 3 (AOM/DSS/2 ppm MEL, 62.9) and group 4 (AOM/DSS/10 ppm MEL, 56.3) were significantly smaller than that of group 1 (AOM/DSS, 80.8). The apoptotic indices measured by TUNEL and ssDNA methods in the morphologically intact colonic mucosa ($n = 5$ for each group) were 2.8 ± 0.8 and 2.0 ± 0.4 in group 1, 3.0 ± 0.7 and 1.8 ± 0.3 in group 2, 2.8 ± 0.8 and 2.2 ± 0.4 in group 3, 2.6 ± 0.5 and 2.8 ± 0.4 in group 4, 2.8 ± 0.8 and 2.4 ± 0.5 in group 5, 6.4 ± 0.9 and 1.9 ± 0.2 in group 6, 9.0 ± 0.7 and 2.3 ± 0.4 in group 7, 2.5 ± 1.0 and 2.6 ± 0.5 in group 8 and 2.4 ± 0.8 and 2.5 ± 0.5 in group 9, respectively. The values were insignificant among the groups. As illustrated in Fig. 3, apoptotic indices determined by the TUNEL (Fig. 3A) – and ssDNA (Fig. 3B) – methods of groups 2 through 4 were significantly lower than those of group 1.

3.6. Scores of NF-κB, TNFα, IL-1β and STAT3 immunohistochemistry

The antibodies against NF-κB, TNFα, IL-1β and STAT3 were positive in the nuclei and/or cytoplasm of adenocarcinoma cells and infiltrated mononuclear cells in the stroma. Intact cryptal cells were also positive, but their intensity was very weak in comparison to that of the cancer cells. The mean scores of NF-κB, TNFα, IL-1β and STAT3 immunohistochemistry in the adenocarcinomas developed are illustrated in Fig. 4. The order of the intensity of immunoreactivity of adenocarcinoma cells was NF-κB > STAT3 > IL-1β > TNF-α.

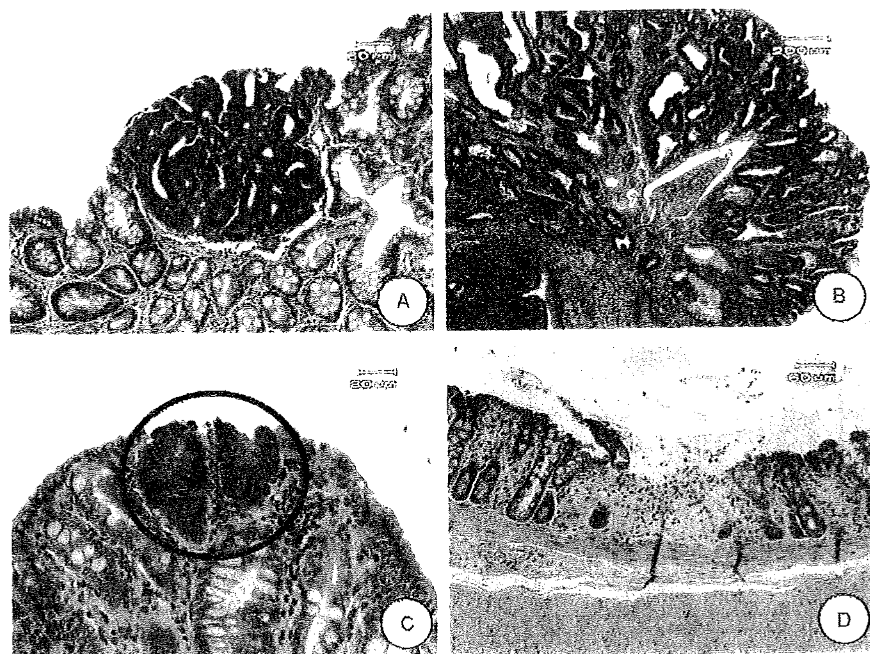

Fig. 1. Representative colonic lesions developed in rats that received AOM/DSS (group 1). (A) A tubular adenoma, (B) a well-differentiated tubular adenocarcinoma, (C) dysplastic crypts (circled) and (D) mucosal ulceration. H & E stain, bars inserted indicate magnification (A, 60 μm; B, 200 μm; C, 30 μm; D, 60 μm).

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

Group no.	Treatment	Number of rats examined	Inflammatory score	Number of mucosal ulcer	Number of dysplasia (high-grade)
1	AOM[a]/2% DSS	25	1.88 ± 0.78[b]	2.08 ± 0.99	11.7[*] ± 3.85
2	AOM/2% DSS + 0.4 ppm MEL	25	0.84 ± 0.62[c]	1.04 ± 0.68	8.08 ± 2.71[c]
3	AOM/2% DSS + 2 ppm MEL	25	0.52 ± 0.65[c]	0.60 ± 0.82[c]	6.24 ± 2.17[c]
4	AOM/2% DSS + 10 ppm MEL	25	0.32 ± 0.48[c]	0.48 ± 0.65[c]	4.72 ± 2.89[c]
5	AOM + 10 ppm MEL	8	0	0	0
6	AOM	8	0	0	0
7	2% DSS	8	1.00 ± 0.54	1.13 ± 0.64	0
8	10 ppm MEL	8	0	0	0
9	Untreated	8	0	0	0

[a] AOM, azoxymethane; DSS, dextran sodium sulfate; MEL, melatonin.
[b] Mean ± S.D.
[c] Significantly different from the AOM/DSS group (group 1) by Turkey–Kramer multiple comparison post-test ($p < 0.001$).

The mean scores of NF-κB (Fig. 4A) of group 2 (AOM/DSS/0.4 ppm MEL, 3.67), group 3 (AOM/DSS/2 ppm MEL, 2.74) and group 4 (AOM/DSS/10 ppm MEL, 1.88) were lower than that of group 1 (AOM/DSS, 4.36). The mean scores of TNF (Fig. 4B)-positivity of group 2 (AOM/DSS/0.4 ppm MEL, 3.00), group 3 (AOM/DSS/2 ppm MEL, 2.32) and group 4 (AOM/DSS/10 ppm MEL, 2.00) were lower than that of group 1 (AOM/DSS, 2.00). The mean positive scores of IL-1β (Fig. 4C) of group 2 (AOM/DSS/0.4 ppm MEL, 2.88), group 3 (AOM/DSS/2 ppm MEL, 2.53) and group 4 (AOM/DSS/10 ppm MEL, 2.12) were lower than that of group 1 (AOM/DSS, 3.92). The mean scores of STAT3 (Fig. 4D) of group 2 (AOM/DSS/0.4 ppm MEL, 2.88), group 3 (AOM/DSS/2 ppm MEL, 2.47) and group 4 (AOM/DSS/10 ppm MEL, 2.31) were lower than that of group 1 (AOM/DSS, 4.24).

3.7. *Clinical chemistry of serum levels of AST, ALT, T-Cho, TG, glucose, leptin, insulin, VLDL, LDL and HDL*

The data on the clinical chemistry of the serum levels of AST, ALT, glucose, leptin and insulin are shown in Fig. 5 and those of T-Cho, TG, VLDL, LDL and HDL in Fig. 6. The administration of MEL dose-dependently lowered the levels of AST (Fig. 5A), ALT (Fig. 5B), insulin (Fig. 5E), T-Cho (Fig. 6A), TG (Fig. 6B), VLDL (Fig. 6C) and LDL (Fig. 6D) when in comparison with that of group 1, but the differences were insignificant. In contrast, MEL exposure at dose levels of 2 and 10 ppm significantly lowered serum levels of glucose (Fig. 5C) and leptin (Fig. 5D). MEL at a dose of 10 ppm significantly elevated the HDL level (Fig. 6E).

4. Discussion

The results presented herein clearly indicated that MEL in drinking water effectively suppresses AOM/DSS-induced rat colitis-related colonic oncogenesis without any adverse effects. The administration of MEL exerted a cancer chemopreventive ability by suppressing several biomarkers [40,41] of colon carcinogenesis. This is the first report showing that prolonged dosing of exogenous MEL exerts cancer chemopreventive ability in colitis-related colon carcinogenesis in rodents. Although other treatment methods of MEL have been previously reported [44], MEL was given in drinking water to rats in this study, as in Sener et al. [45] in order to obtain an effective biological activity of MEL throughout the experiment.

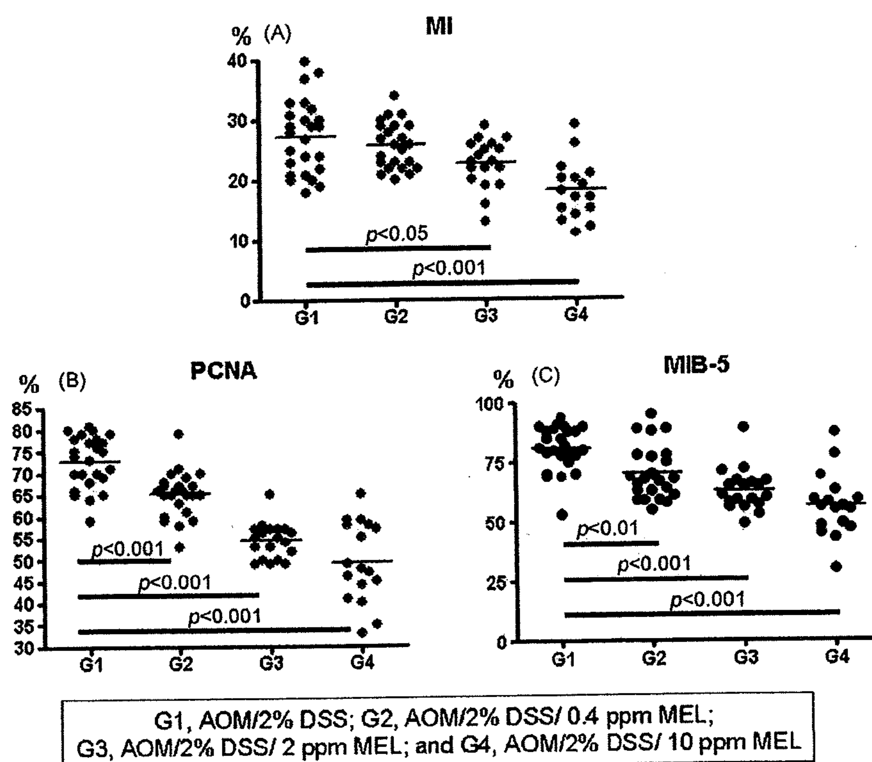

Fig. 2. The mitotic index (MI, panel A) and proliferation indices (PCNA, panel B; MIB-5, panel C) of the adenocarcinomas developed in groups 1 through 4.

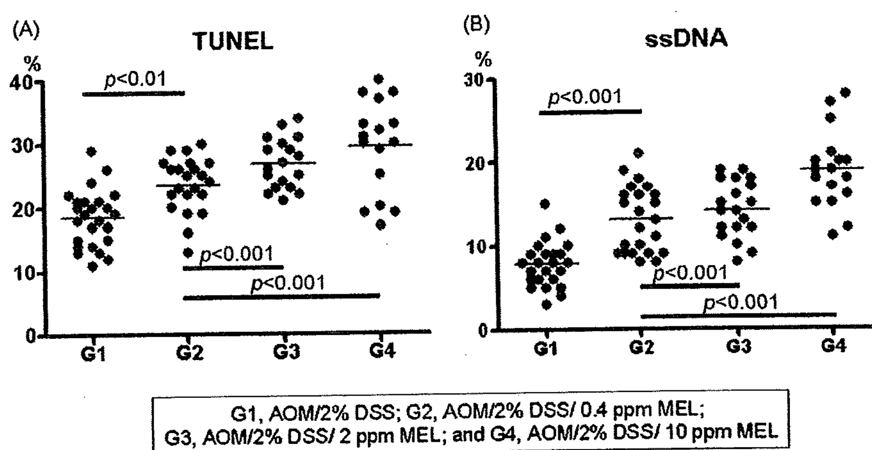

Fig. 3. The apoptotic indices (TUNEL, panel A; ssDNA, panel B) of the adenocarcinomas developed in rats of groups 1 through 4.

Importantly, the 17 weeks administration of MEL (group 8) did not cause any toxic effects in the rats. In this study, the highest dose (10 ppm) of MEL we selected, since that dose inhibits chemically induced colitis in rats [21]. In addition, continuous low doses (0.4, 2 and 10 ppm) of MEL were administered to rats, considering the fact that serum MEL level of adults is about 17 pg/ml at night and the half-life of MEL is about 30 min.

As reported previously in the experiment using mice [26], the incidence and multiplicity of colonic tumors in the rats that received AOM and 2% DSS in the current study were quite high, suggesting that a rat model using AOM and DSS can be utilized to investigate the pathogenesis of colitis-related colon carcinogenesis. The findings presented here also strengthened the importance of inflammation in colonic oncogenesis. In this study, MEL exposure was capable of inhibiting the incidence and multiplicity of colonic epithelial neoplasms (adenoma and adenocarcinoma) together with the frequency of dysplastic crypts in a dose-dependent manner. These findings are important, since the findings that the suppressing effects of MEL on the development of both preneoplasia and neoplasia in the colon suggest the possible application of MEL for clinical use in people at high-risk peoples for CRC.

In the current study, the treatment with MEL in drinking water significantly lowered colonic inflammation induced by DSS. Chronic inflammation is involved in oncogenesis in certain tissues including the large bowel, thus, suppression of chronic inflammation through modulation of expression of several pro-inflammatory gene products that mediate several events of carcinogenesis may result in cancer chemoprevention [46]. MEL has anti-inflammatory

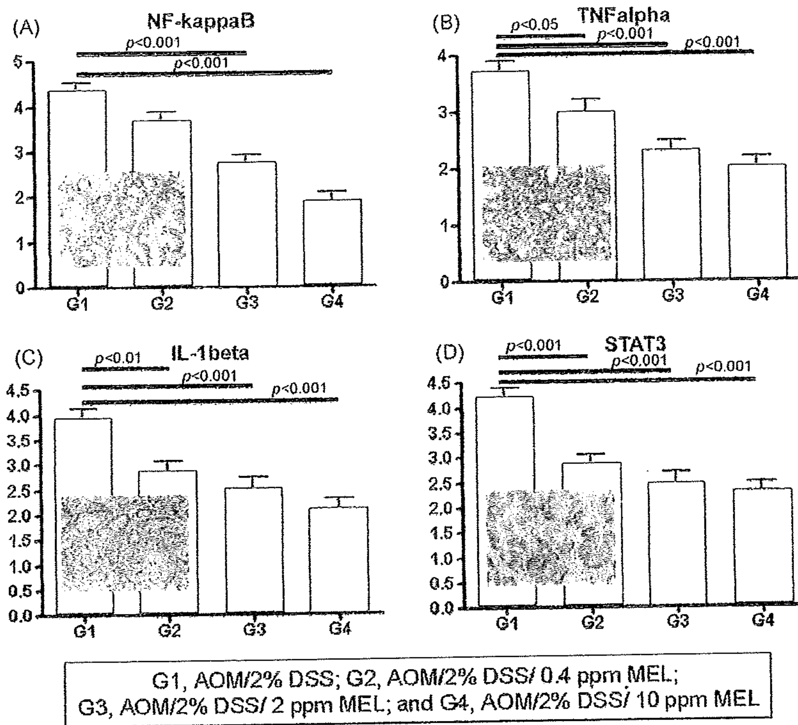

Fig. 4. The immunohistochemical scores of NF-κB (panel A), TNFα (panel B), IL-1β (panel C) and STAT3 (panel D) of the adenocarcinomas developed in rats of groups 1 through 4. Representative immunoreactivity against these antibodies in the cancer cells are inserted. Bars are 30 μm.

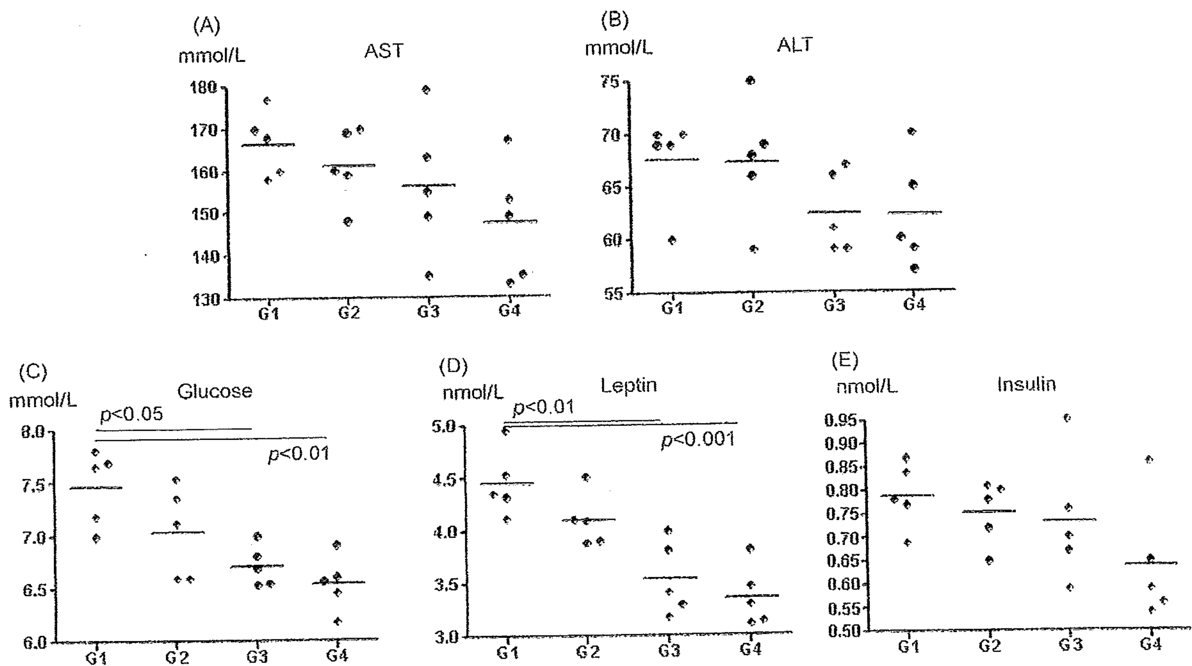

Fig. 5. Serum levels of AST (panel A), ALT (panel B), glucose (panel C), leptin (panel D) and insulin (panel E) of rats in groups 1 through 4.

effects and inhibits iNOS and COX-2 expression [20,47]. The modulation of inflammation and expression of COX-2 and iNOS in the colon results in the suppression of colitis-related colon carcinogenesis of mice [48]. Since several molecular targets for suppression of inflammation-associated carcinogenesis are proposed [49], further studies are warranted to determine the detailed mechanisms by which MEL inhibits inflammation-related carcinogenesis. In this study, MEL treatment modified the expression of NF-κB, TNFα, IL-1β and STAT3. There are candidate biomarkers of colon tumorigenesis [40,41], since the expression of NF-κB, TNFα and IL-1β is involved in colonic tumorigenesis by affecting proliferation and apoptosis [50–53]. In addition, STAT3 expression is an important factor in colon carcinogenesis, tumor invasion [54] and survival/proliferation of colonic preneoplastic cells [55]. In addition,

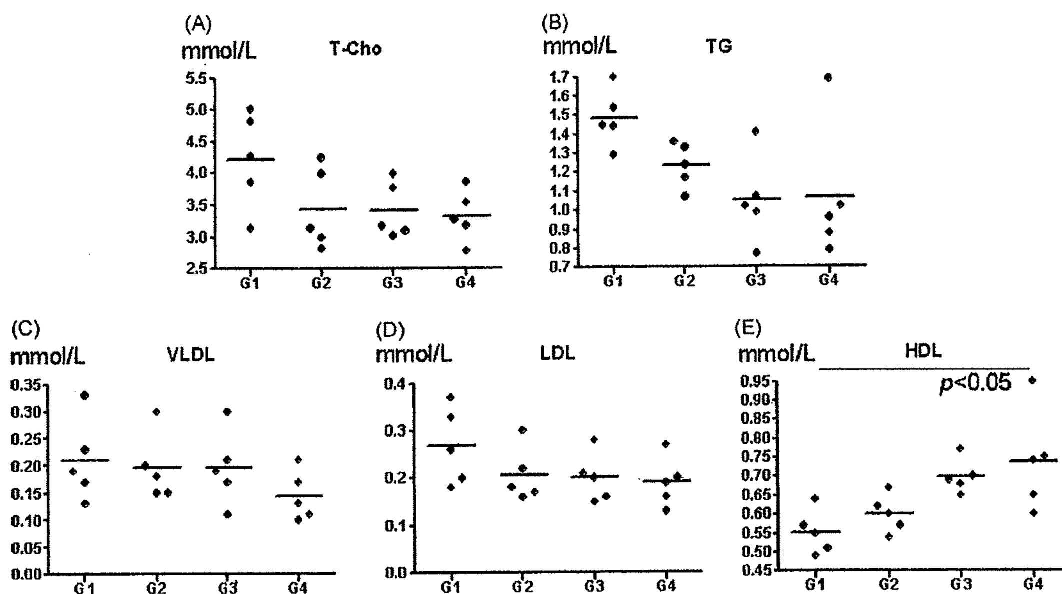

Fig. 6. Serum levels of T-Cho (panel A), TG (panel B), VLDL (panel C), LDL (panel D) and HDL (panel E) of rats in groups 1 through 4.

the anti-inflammatory potential of melatonin through suppression of the expression of NF-κB and chemokines (interleukin-8 and monocyte chemoattractant protein) in a rat colitis model [21,56] is of interest and important for further investigation of cancer chemopreventive ability of this bioactive substance, as observed in this study.

The current study measured the effects of exogenous MEL on the liver function (AST, ALT), lipid profile (T-Cho, TG, VLDL, LDL and HDL) and metabolic alteration (glucose, leptin and insulin). All measurements were the greatest in group 1 that received AOM/DSS, as observed in previous experiments using mice (unpublished work). MEL exposure lowered the serum levels of AST and ALT that were elevated by the treatment with AOM/DSS. Although the differences were insignificant, the findings may suggest the hepatoprotective effects of MEL [57]. In addition, MEL administration influenced the lipid profile. Although the effects on the serum levels of T-Cho, TG, VLDL and LDL were insignificant, an elevation of serum HDL level was observed, as reported by others [45,58]. In addition, MEL lowered the serum levels of glucose and leptin [59,60], thus suggesting the possible application of MEL in the management of obesity and/or diabetes [61].

In conclusion, prolonged exogenous MEL in drinking water was thus found to effectively inhibit colonic cancer development in a two-stage colitis-related rat colon oncogenesis model through modulation of inflammation and proliferation in the inflamed colon of rats that received AOM and DSS. The inhibition by MEL might be mediated by modulating the expression of NF-κB, TNFα, IL-1β and STAT3.

Conflict of interest

None declared.

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Zerumbone, a tropical ginger sesquiterpene, inhibits colon and lung carcinogenesis in mice

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Zerumbone (ZER), present in subtropical ginger *Zingiber zerumbet* Smith, possesses anti-growth and anti-inflammatory properties in several human cancer cell lines. ZER also down-regulates the cyclooxygenase-2 and inducible nitric oxide synthase expression *via* modulation of nuclear factor (NF)-κB activation in cell culture systems. These findings led us to investigate whether ZER is able to inhibit carcinogenesis in the colon and lung, using 2 different preclinical mouse models. In Exp. 1, a total of 85 male ICR mice were initiated using a single intraperitoneal (i.p.) injection with azoxymethane (AOM, 10 mg/kg bw) and promoted by 1.5% dextran sulfate sodium (DSS) in drinking water for 7 days for rapid induction of colonic neoplasms. Animals were then fed the diet containing 100, 250 or 500 ppm ZER for 17 weeks. In Exp. 2, a total of 50 female A/J mice were given a single i.p. injection of 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (10 μmol/mouse) to induce lung proliferative lesions. They were then fed the diet mixed with 100, 250 or 500 ppm ZER for 21 weeks. At the termination of the experiments (wk 20 of Exp. 1 and wk 22 of Exp. 2), all animals were subjected to complete necropsy examination to determine the pathological lesions in both tissues. Oral administration of ZER at 100, 250 and 500 ppm significantly inhibited the multiplicity of colonic adenocarcinomas. The treatment also suppressed colonic inflammation. In the lung carcinogenesis, ZER feeding at 250 and 500 ppm significantly inhibited the multiplicity of lung adenomas in a dose-dependent manner. Feeding with ZER resulted in inhibition of proliferation, induction of apoptosis, and suppression of NFκB and heme oxygenase (HO)-1 expression in tumors developed in both tissues. Our findings suggest that dietary administration of ZER effectively suppresses mouse colon and lung carcinogenesis through multiple modulatory mechanisms of growth, apoptosis, inflammation and expression of NFκB and HO-1 that are involved in carcinogenesis in the colon and lung.
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Key words: zerumbone; colon; lung; carcinogenesis; chemoprevention

A sesquiterpenoid, zerumbone (ZER), is a major constituent of the subtropical ginger plant *Zingiber zerumbet* Smith. The essential oil of the rhizomes contains large amount of ZER and is used as an anti-inflammatory medicine.[1] Recent studies revealed several biological properties of ZER that may be responsible for inhibition of carcinognesis. They include suppression of skin tumor promoter 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-induced Epstein-Barr virus activation in Raji cells,[1] inhibition of free radical generation, inhibition of inducible nitric oxide synthase (iNOS) and cyclooxygenase (COX)-2 expression, inhibition of tumor necrosis factor (TNF)-α-release in activated leukocytes and induction of apoptosis in human colonic adenocarcinoma (ADC) cell lines.[2] *In vivo* studies demonstrated that dietary feeding with ZER markedly suppressed dextran sulfate sodium (DSS)-induced acute colitis in mice[3] and a putative precursor lesions for colonic ADC, aberrant crypt foci (ACF), produced by azoxymethane (AOM) in rat colon.[4] The findings were accompanied by reductions of prostaglandin E_2 and COX-2 protein expression in colonic mucosa.[3,4] Additionally, ZER suppresses the combined lipopolysaccharide- and interferon-γ-induced IκB protein degradation in macrophages.[5] More recently, Takada *et al.*[6] reported that ZER suppresses nuclear factor (NF)-κB activation induced by TNF, okadaic acid, cigarette smoke condensate, TPA and H_2O_2.

Colorectal cancer (CRC) and lung cancer are major epithelial malignancies and both are increasing in developed countries. An association between inflammation and cancer has long been suspected[7] and inflammatory condition is a risk for cancer development in colon and lung.[8–12] A representative example is that inflamed colon has a high risk for CRC development. In patients with inflammatory bowel disease, including ulcerative colitis and Crohn's disease, the risk of CRC development is greater than in the general population.[13] Smoking is a risk factor of development of different types of cancer in different tissues, including lung[14] and colon.[15] Also, inflammation caused by tobacco enhances lung carcinogenesis.[11] Despite well-developed diagnostic and therapeutic techniques, and novel anti-cancer drugs against both malignancies have been introduced, mortality rates of CRC and lung cancer have not remarkably been improved. Therefore, we need some new weapons and strategies for fighting against these malignancies. Cancer chemoprevention is one of such strategies. For clinical use of candidate cancer chemopreventive agents, they need to be determined preclinical efficacy using appropriate animal carcinogenesis models. As to inflammation-associated colon carcinogenesis, we have developed a mouse model utilizing a colon carcinogen AOM and a colitis-inducing agent DSS.[16] 4-(*N*-methyl-*N*-nitrosamino)-1-(3-pyridyl)-1-butanone (NNK)-induced mouse lung tumorigenesis model is frequently used for carcinogenesis and chemoprevention studies, as NNK is a tobacco-specific carcinogenic nitrosamine, which derived from nicotine.[14,17] Hecht *et al.*[18] developed a relatively rapid single-dose model for induction of lung adenomas (ADs) in female A/J mice initiated with NNK. The fact that nonsteroidal anti-inflammatory drugs can inhibit NNK-induced lung tumors[19] suggests that inflammation is involved in NNK-induced lung tumorigenesis.

In the current study, we investigated the chemopreventive ability of ZER in colon and lung carcinogenesis using a mouse colitis-related CRC model[16] and a NNK-induced mouse lung carcinogenesis model.[18] Also, the effects of ZER on the immunohistochemical

Abbreviations: ACF, aberrant crypt foci; AD, adenoma; ADC, adenocarcinoma; AOM, azoxymethane; ARE, antioxidant response element; CRC, colorectal cancer; DAB, 3,3'-diaminobenzidine; DSS, dextran sulfate sodium; GPx, γ-glutamylcystein glutathione peroxidase; HO, heme oxygenase; H&E, hematoxylin and eosin; HP, hyperplasia; iNOS, inducible nitric oxide synthase; MnSOD, manganese superoxide dismutase; NF-κB, nuclear factor-kappaB; NNK, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone; NrF, nuclear factor-erythroid 2-related factor; PCNA, proliferating cell nuclear antigen (PCNA); TNF, tumor necrosis factor; TPA, 12-*O*-tetradecanoylphorbol-13-acetate; TUNEL, TdT-mediated dUTP nick-end labeling; ZER, zerumbone.

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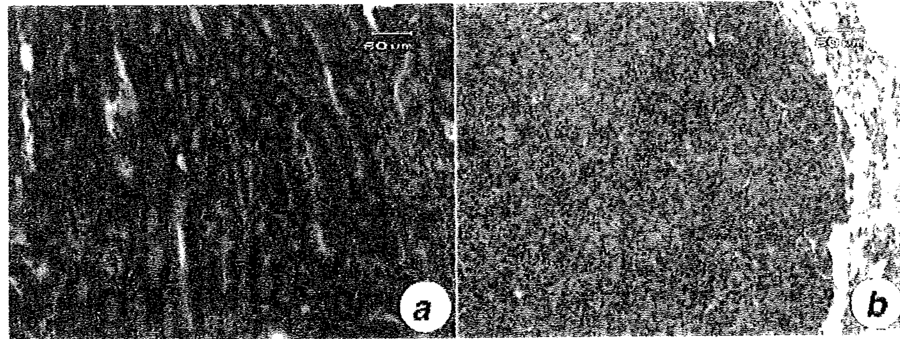

FIGURE 2 – Histopathology of (*a*) colonic tubular ADC induced by AOM/DSS and (b) lung tubular adenoma induced by NNK. Bars in the photos indicate magnification (μm).

TABLE II – EFFECTS OF DIETARY ZERUMBONE ON THE DEVELOPMENT OF COLONIC ADENOMA AND ADENOCARCINOMA (EXP. 1)

Group no.	Treatment	No. of mice examined	Incidence (%)			Multiplicity (no. of tumors/colon)		
			AD	ADC	Total	AD	ADC	Total tumors
1	AOM[1]/DSS	15	60	93	93	1.60 ± 1.55[2]	3.87 ± 2.90	5.47 ± 4.02
2	AOM/DSS + 100 ppm ZER	10	70	70	80	1.60 ± 1.58	1.50 ± 1.51[3]	3.10 ± 2.64
3	AOM/DSS + 250 ppm ZER	10	90	60	90	2.00 ± 1.33	1.20 ± 1.23[4]	3.20 ± 2.39
4	AOM/DSS + 500 ppm ZER	10	50	50[5]	60	0.90 ± 1.10	0.60 ± 0.70[4]	1.50 ± 1.72[3]
5	DSS + 500 ppm ZER	5	0	0	0	0	0	0
6	DSS	5	0	0	0	0	0	0
7	500 ppm ZER	5	0	0	0	0	0	0
8	Untreated	5	0	0	0	0	0	0

[1]AOM, azoxymethane; DSS, dextran sulfate sodium; ZER, zerumbobe; AD, adenoma; and ADC, adenocarcinoma.–[2]Mean ± SD.–[3,4]Significantly different from the AOM/DSS group (group 1) by Tukey-Kramer multiple comparison posttest ($^3p < 0.05$ and $^4p < 0.01$).–[5]Significantly different from the AOM/DSS group (group 1) by Fisher's exact probability test ($p = 0.0225$).

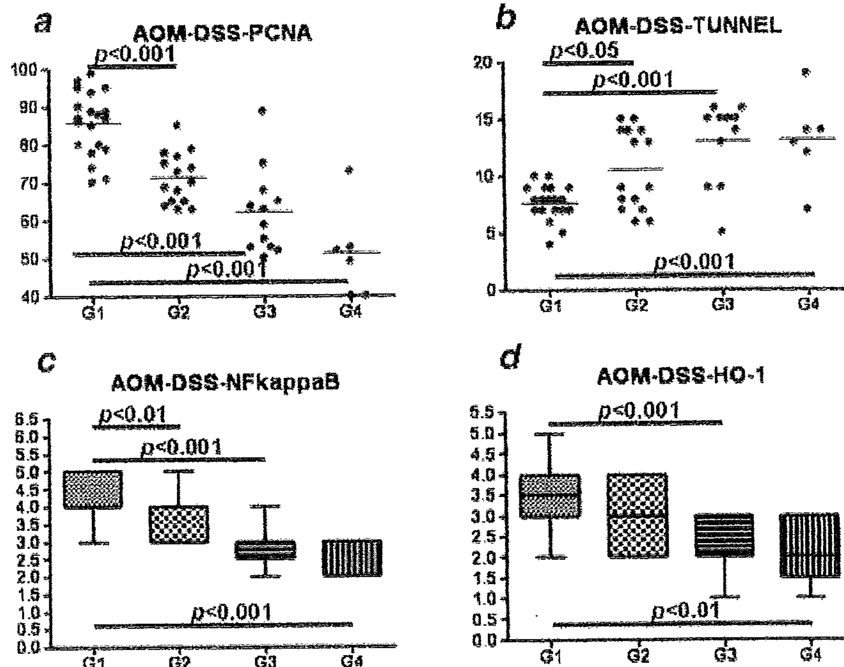

FIGURE 3 – Immunohistochemical scores of (*a*) PCNA-labeling index, (*b*) apoptotic index, (*c*) NF-κB and (*d*) HO-1, which were determined in colonic ADCs developed in mice of groups 1 through 4.

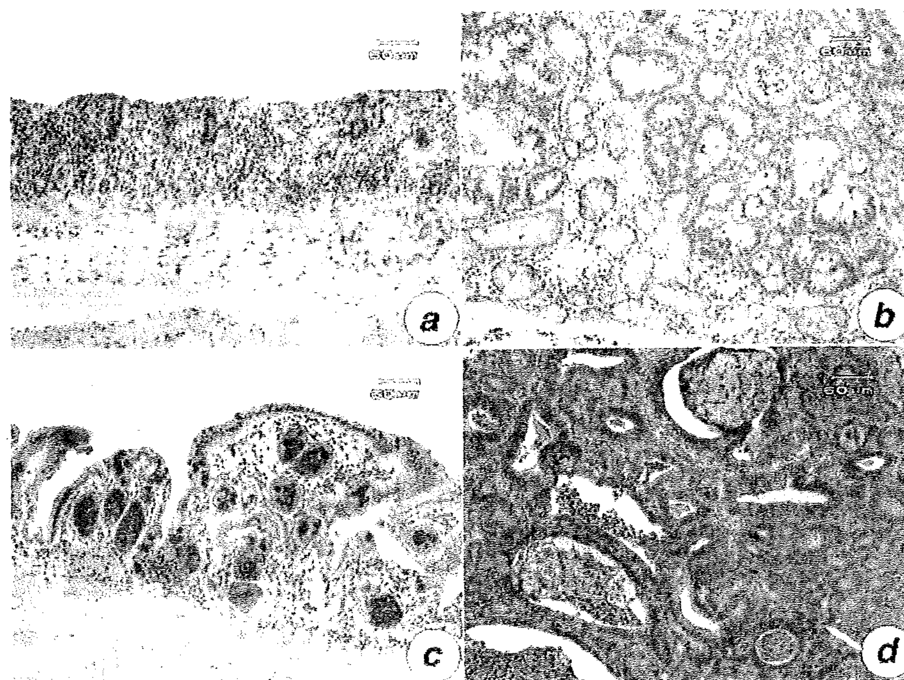

FIGURE 4 – Immunohistochemistry of NF-κB (*a*, *b*) and HO-1 (*c*, *d*). Strong NF-kB expression is observed in (*a*) mononuclear inflammatory cells in the colonic mucosa and (*b*) colonic ADC cells. HO-1 expression is relatively weak in (*c*) mononuclear inflammatory cells in the colonic mucosa and strong in (*d*) some of ADC cells. Bars in the photos indicate magnification (μm).

TABLE III – EFFECTS OF DIETARY ZERUMBONE ON THE DEVELOPMENT OF LUNG PROLIFERATIVE LESIONS INDUCED BY NNK (EXP. 2)

Group no.	Treatment	No. of mice examined	Incidence (%)			Multiplicity (no. of proliferative lesions/lung)		
			HP	AD	Total	HP	AD	Total
1	NNK[1]	12	100[2]	100[2]	100	2.75 ± 0.97[3,4]	8.25 ± 2.83[4]	11.00 ± 2.92[4]
2	NNK+100 ppm ZER	10	70	100	100	1.50 ± 1.18[5]	6.40 ± 2.63	7.90 ± 2.64
3	NNK+250 ppm ZER	10	40[6]	90	90	0.50 ± 0.71[7]	4.70 ± 3.33[5]	5.20 ± 3.74[7]
4	NNK+500 ppm ZER	10	90	80	90	0.90 ± 0.32[7]	2.60 ± 2.17[7]	3.50 ± 2.32[7]
5	500 ppm ZER	5	40	20	40	0.40 ± 0.55	0.20 ± 0.45	0.60 ± 0.89
6	Untreated	5	40	40	60	0.40 ± 0.55	0.60 ± 0.89	1.00 ± 1.22

[1]NNK, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone; HP, hyperplasia; AD, adenoma; and ZER, zerumbone.–[2]Significantly different from the untreated group (group 6) by Fisher's exact probability test ($p = 0.0147$).–[3]Mean ± SD.–[4]Significantly different from the untreated group (group 6) by Tukey-Kramer multiple comparison posttest ($p < 0.001$).–[5]Significantly different from the NNK group (group 1) by Tukey-Kramer multiple comparison posttest ($p < 0.05$).–[6]Significantly different from the NNK group (group 1) by Fisher's exact probability test ($p = 0.0028$).–[7]Significantly different from the NNK group (group 1) by Tukey-Kramer multiple comparison posttest ($p < 0.001$).

Exp. 2: Effect of ZER on NNK-induced lung carcinogenesis

General observation. Any clinical signs of toxicity of dietary ZER were not noted during the experiment. At sacrifice, the mean weight of lungs of the NNK-treated mice (group 1, 0.45 ± 0.05 g, $p < 0.05$) was significantly greater than that of the untreated mice (group 6, 0.35 ± 0.02 g). The mean weight of lungs of the mice in groups 2 (0.39 ± 0.05 g, $p < 0.05$), 3 (0.35 ± 0.05 g, $p < 0.05$), and 4 (0.36 ± 0.03 g, $p < 0.05$) were significantly lower as compared with that of group 1. Other measures (body, liver, kidney and spleen weights) did not significantly differ among the groups.

Effects of dietary ZER on the development of lung proliferative lesions. Table III summarizes the data on the incidence and multiplicity of lung proliferative lesions (HP and AD) induced by NNK and/or ZER. All mice belonging to group 1 developed alveolar cell HP and AD (Fig. 2*b*) with 100% incidences ($p = 0.0147$ for each) with high multiplicities of HP (2.75 ± 0.97, $p < 0.001$) and AD (8.25 ± 2.83, $p < 0.001$), as compared with an untreated group (group 6). Dietary ZER slightly affected the incidences of lung HP and AD, but significantly lowered the multiplicity of HP (100 ppm ZER: 1.50 ± 1.18, $p < 0.05$; 250 ppm ZER: 0.50 ± 0.71, $p < 0.001$; and 500 ppm ZER: 0.90 ± 0.32, $p < 0.001$) as compared group 1. Likewise, the supplementation of ZER at 250 (4.70 ± 3.33, $p < 0.05$) and 500 ppm (2.60 ± 2.17, $p < 0.001$) to the diet significantly reduced the multiplicity of AD when compared to group 1. The inhibition by 100 ppm ZER feeding was insignificant. Suppression effects of ZER at 3 dose levels demonstrated an inverse relationship of inhibition in the multiplicity of lung AD (Pearson $r = -0.9855$, $p < 0.00145$).

Effects of ZER on proliferation and apoptosis of lung ADs. As shown in Figure 5, ZER feeding at 250 ppm ($p < 0.01$) and 500 pmm ($p < 0.001$) significantly decreased the PCNA-labeling index of adenoma cells (Fig. 5*a*) and significantly increased TUNEL-positive apoptotic nuclei ($p < 0.01$ at 100 and 250 ppm, and $p < 0.001$ at 500 ppm) of lung adenoma cells (Fig. 5*b*).

Immunohistochemical scores of NFκB and HO-1 in lung ADs. Positive immunohistochemical reactions of NFκB (Fig. 6*a*) and HO-1 (Fig. 6*c*) were observed in lung adenomas that developed in NNK-treated mice. The positive reactions were reduced in

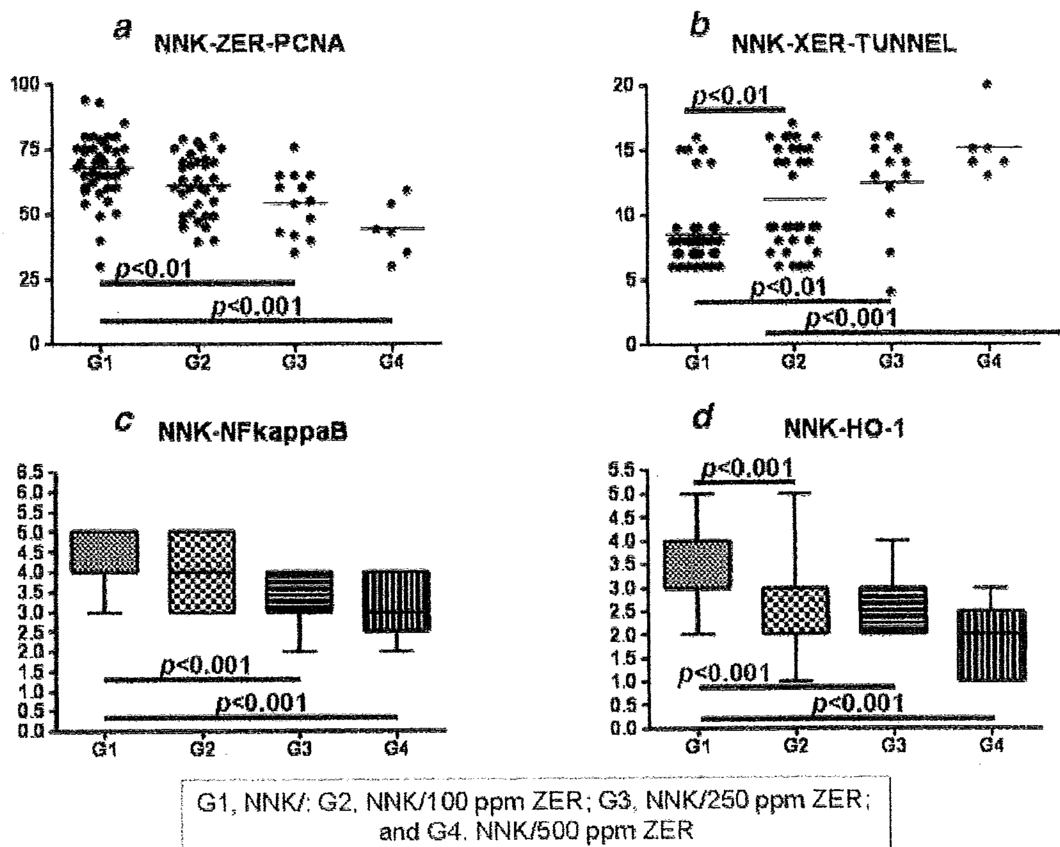

FIGURE 5 – Immunohistochemical scores of (*a*) PCNA-labeling index, (*b*) apoptotic index, (*c*) NF-κB and (*d*) HO-1, which were determined in lung adenoma developed in mice of groups 1 through 4.

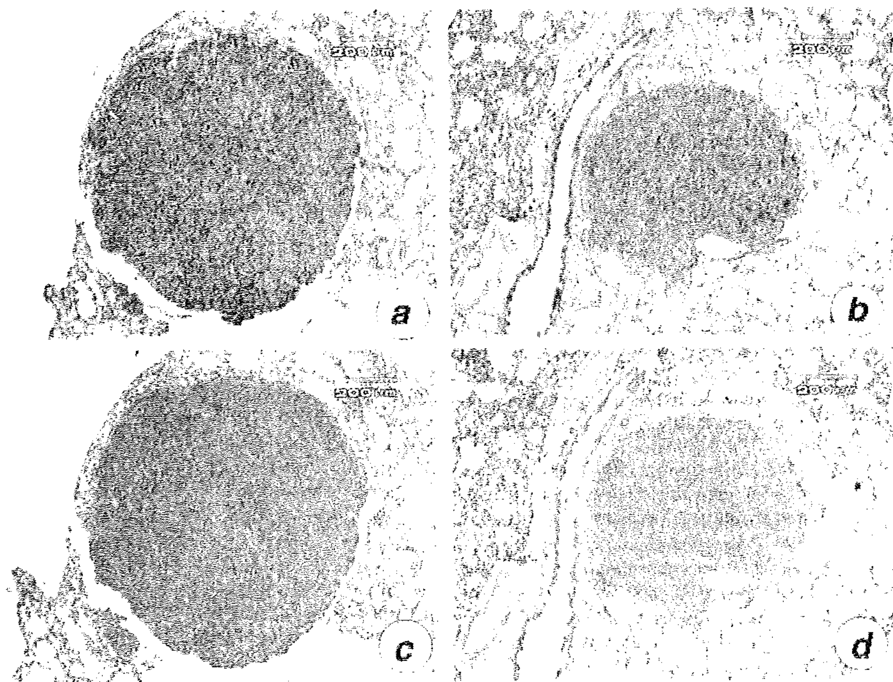

FIGURE 6 – Representative immunohistochemical reactions of NF-κB (*a*, *b*) and HO-1 (*c*, *d*) of lung adenomas. While strong reactivities of (*a*) NF-κB and (*c*) HO-1 are observed in the lung adenoma from group 1 (NNK alone), those of (*c*) NF-κB and (*c*) HO-1 from group 4 (NNK + 500 ppm ZER) are weak.

the lung AD (Figs. 6*b* and 6*d*) of mice that received NNK and ZER. As illustrated in Figure 5, feeding with ZER dose-dependently reduced the immunohistochemical scores and the inhibition of the NF-kB score (Fig. 5*c*) by ZER at 250 ($p < 0.001$) and 500 ppm ($p < 0.001$) and that of the HO-1 score (Fig. 5*d*) by ZER at 100 ($p < 0.001$), 250 ($p < 0.001$) and 500 ppm ($p < 0.001$) were statistically significant.

Discussion

The findings described here clearly indicated the chemopreventive effects of ZER derived from wild ginger in the mouse colon and lung carcinogenesis models. The protective ability of ZER is considered to be mediated by its anti-proliferative, apoptosis-inducing, anti-inflammatory and suppression of NF-kB and HO-1 expression. Together with our previous findings,[4,28] ZER is one of the possible chemopreventive agents against carcinogenesis in different tissues, including colon, lung and skin.[28] As large amount of ZER is readily available from the rhizomes of *Z. zerumbet*, further investigations for clarifying for detailed mechanisms of inhibition can be conducted in multiple organs of preclinical and clinical chemopreventive studies.

We assessed in this study chemopreventive ability of ZER at 3 dose levels (100, 250 and 500 ppm in diet) using 2 different mouse carcinogenesis models. All doses of ZER suppressed colonic inflammation and reduced the multiplicity of colonic ADC formation induced by AOM/DSS in a dose-dependent manner. In the NNK-induced lung tumorigenesis, ZER at all doses reduced the multiplicity of proliferative lesions, HP and ADs. The suppressing effects of ZER on the multiplicity of lung ADs showed clear dose-dependency. Importantly, we did not observe any toxicity of ZER in 2 different experiments.

As expected from our previous study[4] showing the inhibitory effects of ZER on the development of AOM-induced ACF, which is putative precursor lesion for colonic ADCs,[29,30] dietary ZER inhibited the occurrence of colonic ADC induced by the AOM/DSS treatment. Although the model system of colitis-related colon carcinogenesis[16] used in this study to induce colonic preneoplastic and neoplastic lesions was different from that for sporadic CRC,[4,29,30] protective effects of ZER on colon tumorigenesis are more likely in the inflamed colon, where the risk for ADC development is quite high.[31] Previous *in vitro* and *in vivo* investigations revealed strong anti-inflammatory properties of ZER.[1–3,5,28,32,33] Moreover, ZER can induce detoxifying enzymes.[34] ZER, thus, might be a cancer chemopreventive agent for the high-risk groups for CRC, such as ulcerative colitis and patients who receive polypectomy or surgical resection of CRC.

NF-κB is the key transcriptional factor for synthesis of proinflammatory mediators, including iNOS, COX-2 and TNF-α. NF-κB also plays central roles in carcinogenesis and inflammation,[21] and thus it is one of the molecular targets of cancer chemoprevention and therapy.[24,35] In fact, NF-κB activation is reported to be involved in colon[36] and lung carcinogenesis[37] and certain NF-κB inhibitors are able to suppress cancer development in these tissues.[38,39] In this study, dietary administration of ZER reduced the immunohistochemical expression of NFκB in colonic and lung tumors. Also, we observed that ZER causes suppression of cell proliferation, and induction of apoptosis. ZER is reported to suppress proinflammatory protein production and oxidative/nitrosative stress, and to induce apoptosis in human colon cancer cell lines *via* suppressing the expression of COX-2 and iNOS.[2] ZER induces nuclear localization of nuclear factor-erythroid 2-related factor (Nrf)-2 that binds to antioxidant response element (ARE) of the phase II enzyme genes, suggesting that ZER is a potential activator of the Nrf-2/ARE-dependent phase II enzyme genes, including manganese superoxide dismutase (MnSOD), γ-glutamylcystein glutathione peroxidase (GPx) 1[28] and HO-1.[34] In the current study, dietary feeding with ZER inhibited the immunohistochemical expression of HO-1 in the tumors developed in the colon and lung. Both *MnSOD* and *GPx1* are regulated, at least in part, by Nrf-2 transcription factor.[40] Therefore, ZER may induce these genes' expression *via* possibly Nrf-2 activation An anti-oxidative enzyme, HO-1, is protective against oxidative stress in the damaged tissues without neoplastic alterations.[41] However, overexpression of HO-1 is observed in preneoplastic[42] and neoplastic tissues[43–45] to growth and to survive against cancer therapy.[44] In this context, the effects of ZER on the expression of NF–κB and HO-1 in tumors developed in the colon and lung are of interest. We observed that dietary ZER effectively inhibits immunohistochemical expression of colonic ADCs and lung AD, suggesting that ZER has cancer chemopreventive as well as cancer chemotherapeutic potentials.

In the current experiments, we noted that ZER treatment induces apoptosis in the neoplasms of colon and lung. ZER is previously reported to induce apoptosis in a variety of colon cancer cell lines (LS174, LS180, COLO205 and COLO320DM) with different degree.[2] The α,β-unsaturated carbonyl group of ZER is suspected to be responsible for the effects.[2] A recent report by Sakinah *et al.*[46] showing that ZER treatment results in decreased expression of the anti-apoptotic protein Bcl-2 and increased the expression of the pro-apoptotic protein Bax in human hepatocellular cancer cells, HepG2, confirmed apoptosis-inducing effects of ZER in malignant epithelial cells.

In conclusion, our findings indicate that a sesquiterpenoid, zerumbone, being a major constituent of the subtropical ginger plant *Zingiber zerumbet* Smith is one of the good candidates with multiple targets for cancer chemopreventive agent in colon and lung carcinogenesis related with inflammation.

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Cancer **Prevention** Research

Dietary Tricin Suppresses Inflammation-Related Colon Carcinogenesis in Male Crj: CD-1 Mice

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Abstract The flavone 4′,5,7-trihydroxy-3′,5′-dimethoxyflavone (tricin) present in rice, oats, barley, and wheat exhibits antigrowth activity in several human cancer cell lines and anti-inflammatory potential. However, the chemopreventive activity has not yet been elucidated in preclinical animal models of colorectal cancer. This study was designed to determine whether dietary tricin exerts inflammation-associated colon carcinogenesis induced by azoxymethane and dextran sulfate sodium in mice. Male Crj: CD-1 mice were initiated with a single i.p. injection of azoxymethane (10 mg/kg body weight) and followed by a 1-week exposure to dextran sulfate sodium (1.5%, *w/v*) in drinking water to induce colonic neoplasms. They were then given the experimental diet containing 50 or 250 ppm tricin. The experiment was terminated at week 18 to determine the chemopreventive efficacy of tricin. In addition, the effects of dietary tricin on the expression of several inflammatory cytokines, including tumor necrosis factor (TNF)-α, were assayed. The development of colonic adenomas and adenocarcinomas was significantly reduced by feeding with 50 and 250 ppm tricin, respectively. Dietary tricin also significantly reduced the proliferation of adenocarcinoma cells as well as the numbers of mitoses/anaphase bridging in adenocarcinoma cells. The dietary administration with tricin significantly inhibited the expression of TNF-α in the nonlesional cypts. Our findings that dietary tricin inhibits inflammation-related mouse colon carcinogenesis by suppressing the expression of TNF-α in the nonlesional cyrpts and the proliferation of adenocarcinomas suggest a potential use of tricin for clinical trials of colorectal cancer chemoprevention.

Cancer mortality rates in the developed countries have increased throughout this century, and has been already the leading cause of death in some Western countries (1, 2). Great advances have been made in the pharmacologic-based treatment of malignant epithelial malignancies. There has also been a marked increase in the understanding of cell and molecular mechanisms underlying a variety of carcinogenic processes (3). However, therapeutic options for advanced neoplastic disease remain limited. This lack of treatment alternatives may be due to the large number of genetic and molecular alterations associated with advanced neoplasms that contribute to the maintenance of neoplastic progression.

The chemopreventive approach to inhibit cancer development and progression is highly attractive. Practical limitations may exist with respect to developing novel and effective chemopreventive agents through the use of appropriate animal models for preclinical evaluation of candidate chemopreventive agents (4). Some herbal and botanical products that contain flavonoids are likely to possess cancer preventive activities (5). A diet rich in fruits and vegetables has long been suggested to correlate with a reduced risk of certain epithelial malignancies, including cancers in the colon, lung, prostate, oral cavity, and breast (5–7). A number of agents have been reported to be candidate *chemo-inhibitors* of cancer development in various tissues, including colon. Among these agents are the flavonoids, a group of phenolic compounds with structural formula of diphenyl-propane and secondary metabolites produced by plants (5, 8, 9).

4′, 5, 7-Trihydroxy-3′, 5′-dimethoxyflavone (tricin; Fig. 1A) is a flavone, a subgroup of the flavonoid group, which is found *in* rice, oats, barley, and wheat (10). Although the physiologic function of tricin in plants is not well defined, the compound is thought to be produced by the plant during times of environmental stress or pathogenic attack (11) and exert potential allelopathic effects (12). Evidence for the biological activity of tricin in rodents has recently been reported. These biological activities

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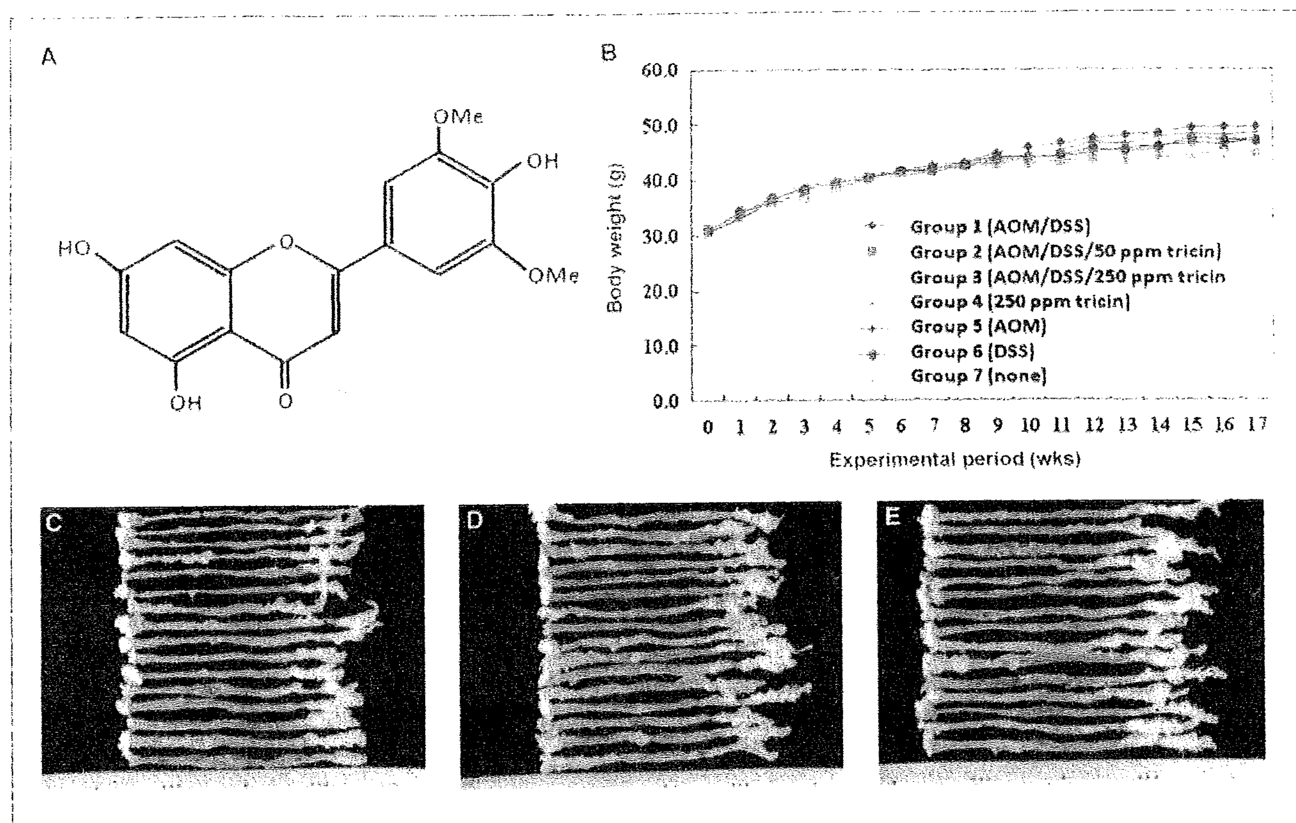

Fig. 1. *A*, the structure of tricin; molecular weight, 330.074. *B*, body weight changes of mice in all groups during the study. Dietary tricin (groups 2, 3, and 5) did not significantly affect the body weight gain. Macroscopic views of the colons from the mice of groups 1 (*C*), 2 (*D*), and 3 (*E*), which received AOM/DSS, AOM/DSS/50 ppm tricin, and AOM/DSS/250 ppm tricin, respectively, at the end of the study (week 18). Although a number of colonic tumors were observed in the mice of group 1, the numbers of the tumors found in groups 2 and 3 were smaller than that in group 1.

include antioxidative (13, 14), antiinflammatory, antiviral (15), and antihistamic (16) activities. These same biological activities have been observed in other promising cancer chemopreventive agents (17–20). The effects of tricin on oncogenesis have been investigated by Gescher et al. Their studies have shown that tricin suppresses the growth of human malignant breast tumor in nude mice (21). Dr. Gescher's group also reported that treatment with tricin-containing extracts from brown rice inhibit the proliferation of human colon and breast cancer cells *in vitro* (22). There are few reports on the effects of dietary tricin on intestinal carcinogenesis. Cai et al. (23) reported that feeding a diet containing 0.2% tricin decreased the size and the number of intestinal adenoma formed in $Apc^{Min/+}$ mice through the inhibition of cyclooxygenase (COX)-2 (23, 24). Dietary tricin did not affect tumor formation in the large bowel (23). Because the concentration of tricin in the mouse intestine is greater than the concentration in the plasma or liver when mice are fed diets containing tricin (25–28), we hypothesized that dietary tricin may affect and possibly inhibit chemically-induced colon carcinogenesis in rodents.

The current study was designed to explore the possible cancer chemopreventive efficacy of tricin. We investigated the effects of dietary tricin on large bowel oncogenesis using an azoxymethane (AOM)/dextran sodium sulfate (DSS)–treated mouse model, which is a useful animal model to study chemoprevention in inflammation-related colon carcinogenesis (29–34). The effects of dietary tricin on the expression of inflammatory enzymes, such as COX-2 (35–37) and inducible nitric oxide synthase (iNOS; refs. 37, 38), and inflammatory cytokines, such as tumor necrosis factor (TNF)-α, (39, 40) NF-κB (17, 40), inhibitor κB (IκB)α, and IκB kinase (IKK)β in the nonlesional colonic mucosa were examined to understand the mechanism(s) by which the compound modify AOM/DSS-induced colon carcinogenesis. In addition, we determined whether dietary tricin affects the chromosomal instability (41) of adenocarcinoma cells by counting the number of anaphase-bridging formations.

Materials and Methods

Chemicals

Tricin (>99% pure confirmed by high performance liquid chromatography) was isolated and prepared from the leaves of *Sasa albo-marginata* (Hououdou Co. Ltd.) by one (M.K.) of the authors (15). In brief, the dried leaves (50 kg) were combined with water (1,000 l) and extracted at 170°C over a period of 3 h. The extracted solution was filtered. The hot water extract of *Sasa albo-marginata* was fractionated successively with ethyl acetate and *n*-butanol. The ethyl acetate fraction (52.0 g) was fractionated using a silica gel 60 (Cica-reagent, 40-50 μm) column (inner diameter 6 × 50 cm, 500 g) and washed with *n*-hexane-ethyl acetate and methanol. This process yielded seven fractions (A-G). Chloroform was added to fraction F (1.50 g) to obtain a chloroform-soluble fraction and an insoluble fraction (solid phase). Tricin (10.0 mg) was recrystallized from the chloroform-soluble fraction as yellow,