

Research Article

Chemopreventive Effects of Frondanol A5, a *Cucumaria frondosa* Extract, against Rat Colon Carcinogenesis and Inhibition of Human Colon Cancer Cell Growth

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Abstract

Sea cucumber extracts have been widely used to treat individuals with inflammatory conditions in East Asia. The present study has been designed to test potential colon cancer-preventive properties of Frondanol A5, a glycolipid extract from the sea cucumber, *Cucumaria frondosa*, using *in vivo* and *in vitro* models of colon cancer. Chemopreventive efficacy of Frondanol A5 was evaluated on azoxymethane-induced rat colon carcinogenesis using colonic aberrant crypt foci (ACF) as efficacy marker. At 7 weeks of age, groups of rats (12 per group) were fed the AIN-76A diet, and ACFs were induced by azoxymethane (15 mg/kg body weight). Three days after azoxymethane treatment, rats were fed with the diets containing 0, 150, and 450 ppm of Frondanol A5 and continued on the diets for 8 weeks, at which time ACFs were evaluated. Expression levels of proliferating cell nuclear antigen and p21^{WAF1/CIP1} were determined in ACFs. Further, Frondanol A5 (10-120 µg/mL) was studied for its growth-inhibitory and apoptotic effects in the HCT-116 cell line. Dietary administration of 150 and 450 ppm of Frondanol A5 significantly suppressed azoxymethane-induced total colonic ACF formation, approximately 34% to 55% ($P < 0.01$ to $P < 0.0001$), and multicrypt aberrant foci (48-68.5%, $P < 0.0001$) in a dose-dependent manner. ACFs in rats treated with Frondanol A5 showed significant upregulation of p21^{WAF1/CIP1} and down-regulation of proliferating cell nuclear antigen compared with control group. Frondanol A5 showed growth inhibition at S and G₂-M phase with a decrease in Cdc25c and an increase in p21^{WAF1/CIP1} with significant apoptosis associated with H2AX phosphorylation and caspase-2 cleavage in HCT116 cells. Overall, Frondanol A5 exhibits potential chemopreventive properties for colon carcinogenesis, which suggests further development of this sea cucumber extract. *Cancer Prev Res*; 3(1); 82-91. ©2010 AACR.

Introduction

Colon cancer is the third leading cause of cancer mortality in the United States for men and women (1). Most colon carcinomas, besides their genetic familial background, develop as a result of multiple assaults by various exogenous/endogenous carcinogenic compounds. It is well established that colon cancer may be ameliorated when risk factors such as genetic, life-style, and environmental factors are modified. Environmental factors, especially food, have been epidemiologically shown to be closely associated with human colorectal cancer (2). Although complete

removal of causative agents of cancer may not always be possible, chemoprevention of cancer using antimutagens and anticarcinogens present in foods or natural products has been suggested by various studies to offer the most effective means of human colon cancer prevention.

Presently, ~60% of drugs approved for cancer treatment are of natural origin (3-5). Large numbers of antimutagens and anticarcinogens that exist in natural products of marine animal and plants inhibit one or more stages of carcinogenesis and prevent or delay cancer development (6). There is an immense diversity of marine plants and animals from which an estimated 14,000 pharmacologically active compounds have been isolated. The marine environment is therefore a rich source for discovering novel lead compounds for the development of new anticarcinogenic drugs (7, 8) and cancer-preventive nutraceuticals. A comprehensive survey of pharmacologic activity was conducted by the U.S. National Cancer Institute over a period of 15 years. The survey found that 4% of the marine species (mainly animals) examined contained antitumor compound(s), a figure close to that was found for terrestrial species (mainly plants) as well. Marine echinoderms are a potential source for new types of biologically active

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compounds with biomedical applications. One of the bioactive compounds is Frondanol A5, a glycolipid extract isolated from the sea cucumber, *Cucumaria frondosa*. The extract has been shown to have a significant inhibitory activity against pancreatic cancer in laboratory animals (9), rat tracheal transformation *in vitro*, and inhibitory activity on the inflammatory markers cyclooxygenase-2 and 5-LOX (10).

Sea cucumber has been valued as a Chinese food and medicine for hundreds of years and as a cure for a wide variety of ailments. Recently isolated compounds from sea cucumbers have antitumor, antiviral, anticoagulant, and antimicrobial activity (11). Sea cucumbers also contain compounds such as Ω -3 fatty acids and fucosylated chondroitin sulfate that are thought to dampen inflammatory conditions in mammals (12). Dried sea cucumbers, in encapsulated form, are available over the Internet as a dietary supplement in the United States and Canada (13). No studies testing these compounds in animals or humans in colon cancer models have thus far been reported in available scientific publications. Hence, Frondanol A5, a glycolipid extract of sea cucumber, was tested in azoxymethane-induced colon carcinogenesis in F344 rats to study the chemopreventive efficacy of Frondanol A5 using colonic aberrant crypt foci (ACF) as efficacy marker. ACF is a reasonable biomarker of colon cancer in preclinical models, and furthermore, in humans, ACF show increased expression of markers of proliferation [proliferating cell nuclear antigen (PCNA)] and inflammation. Therefore, expression levels of a cell proliferating marker (PCNA) and a cell signaling marker (p21^{WAF1/CIP1}) were determined in colonic ACF of treated and control group rats. Further, to assess the possible mechanisms, Frondanol A5 was studied for its growth inhibition and induction of apoptosis in the human colon cancer HCT116 cell line.

Materials and Methods

In vivo studies

Animals, diet, and care. All animal experiments were done in accordance with the institutional guidelines of the American Council on Animal Care. Azoxymethane (NCI # 0061) was from the National Cancer Institute Chemical Carcinogen Reference Standard Repository (Midwest Research Institute). Frondanol A5 was supplied by Coastside Bio Resources. It is an isopropyl alcohol/water extract of the enzymatically hydrolyzed epithelia of the edible North Atlantic sea cucumber, *Cucumaria frondosa*. It also contains several anticancer and anti-inflammatory agents, including monosulphated triterpenoid glycoside Frondoside A, the disulphated glycoside Frondoside B, the trisulphated glycoside Frondoside C, 12-methyltetradecanoic acid, eicosapentaenoic acid and fucosylated chondroitin sulfate, and canthaxanthin/astaxanthin (Fig. 1A). Sulindac was obtained from the Sigma-Aldrich. Weanling male F344 rats were received from the Harlan Breeding Laboratories. All ingredients of the semipurified diet were purchased from BioServ. Male F344 rats were

housed in ventilated cages under standardized conditions (21°C, 60% relative humidity, 12-h light/12-h dark cycle, 20 air changes/hour) in the University of Oklahoma Health Sciences Center barrier facility and were fed a standard laboratory rodent chow and drinking water until initiation of the experiment. Modified AIN-76A diet containing 5% corn oil by weight was prepared (American Institute of Nutrition). Frondanol A5 (150 or 450 ppm) and sulindac (160 ppm) were premixed with a small quantity of casein and then blended into the bulk diet using a Hobart Mixer. In the present study, we used sulindac as positive control. Previously, we have shown that sulindac significantly suppresses the colonic ACF and colonic tumor growth in this model. Both control and experimental diets were prepared weekly and stored in a cold room. Agent content in the experimental diets was determined periodically in multiple samples taken from the top, middle, and bottom portions of individual diet preparations to verify uniform distribution. Rats were allowed *ad libitum* access to the respective diets and automated tap water purified by reverse osmosis.

Bioassay and quantification of ACF. At 7 wk of age, the rats scheduled to receive carcinogen treatment were injected s.c. with azoxymethane at a dose of 15 mg/kg body weight, once weekly for 2 successive wk. Rats intended for vehicle treatment received an equal volume of normal saline. Three days after the final azoxymethane treatment, the rats (12 per group) in the study had access to their respective experimental diets containing 0, 150, or 450 ppm Frondanol A5 or 160 ppm sulindac and continued on the diets for 8 to wk before being sacrificed. All rats were killed by asphyxiation with CO₂ and were necropsied. For ACF quantification, the colons were slit open lengthwise from the anus to the cecum and then fixed flat with mucosa on the upper side between filter papers in 10% buffered formalin (14). After staining with 0.2% methylene blue for 5 min, the mucosal side was observed at $\times 40$ magnification. The total number of ACF in the entire colon was determined in every 2-cm section of the colon, starting from the distal (taken as 0 cm) to the proximal end. ACFs were distinguished from surrounding normal crypts by their longitudinal opening, increased size, intense staining, and pericryptal zone. All colons were scored blindly by two observers and the results were averaged. The parameters used to assess the aberrant crypts were occurrence and multiplicity. Aberrant crypt multiplicity was determined as the number of crypts in each focus and categorized as containing four or more aberrant crypts/focus.

Immunohistochemical staining. Colonic tissue specimens were fixed flat in 10% buffered formalin. The mucosa was stained with 0.2% methylene blue and ACF were identified using a $\times 40$ dissecting microscope. Portions of colonic tissues containing ACFs were paraffin embedded and sectioned for immunohistochemistry. Briefly, for PCNA and p21 immunohistochemical staining, paraffin sections were deparaffinized in xylene, rehydrated through graded ethanol solutions to distilled water, and washed in PBS. Antigen

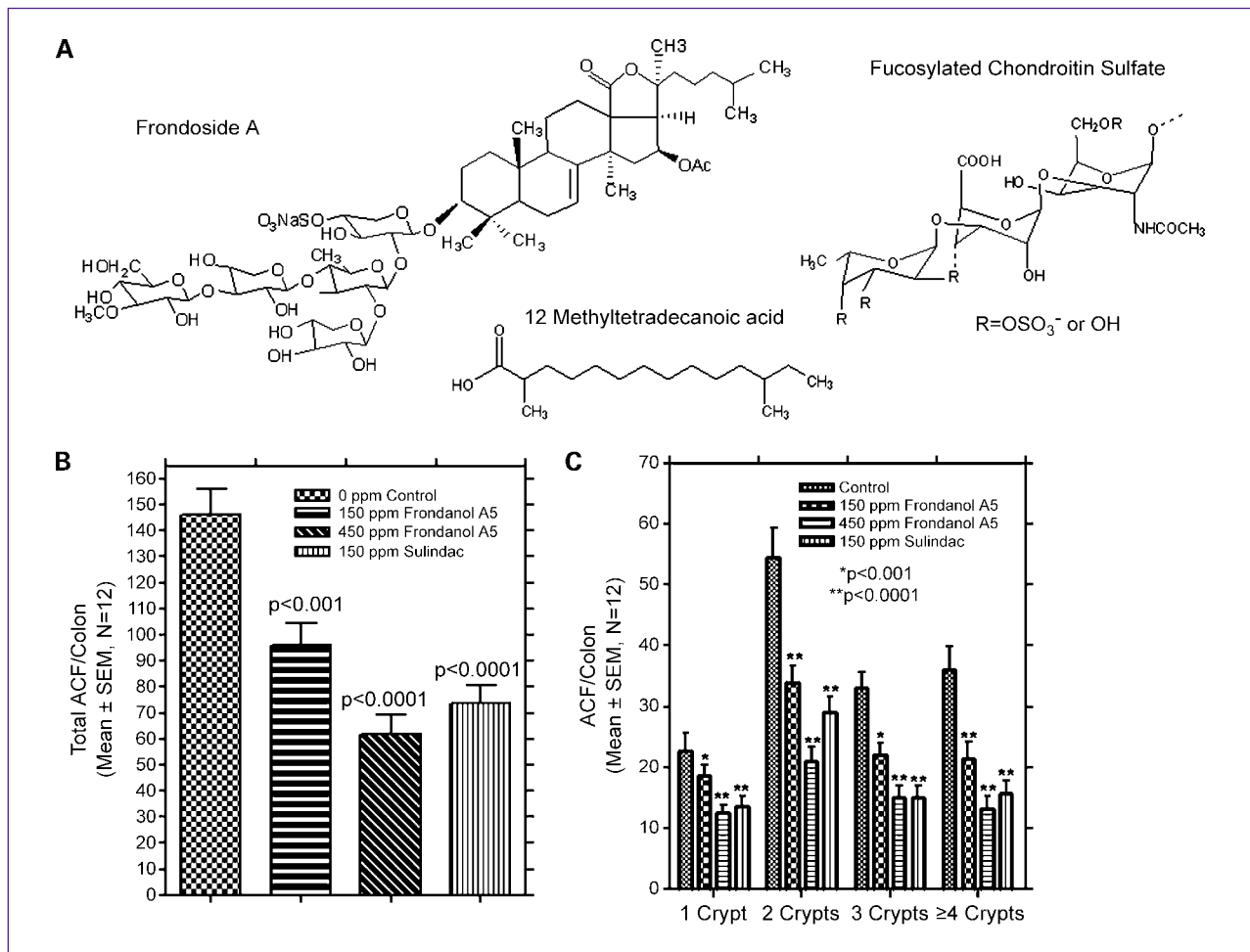


Fig. 1. A, structures of major components of Frondanol A5 present in a glycolipid extract from the sea cucumber. *Frondoside A*, the structure is characterized by the presence of a branched oligosaccharidic chain composed of five units. The holostane-type aglycone features an endocyclic double bond at position C-7,8 and a P-acetoxy group at C-16. *12 Methyltetradecanoic acid*, a branched-chain fatty acid. *Fucosylated chondroitin sulfate*, chemical layout showing one building block of chondroitin that can be repeated numerous times; this is basically a glucose molecule (*left portion*) and glucosamine molecule (*right portion*), which has been sulfated (O_3S , *top*). B, effect of Frondanol A5 on azoxymethane-induced, total mean of colonic ACF formation in F344 rats. C, effect of Frondanol A5 on azoxymethane-induced mean of colonic ACF, which includes one, two, three, and four or more crypt ACF formation in F344 rats. Frondanol A5 showed significant inhibition of one, two, three, or four or more crypt ACF compared with control groups. Specifically a significant ($P < 0.0001$) inhibition of two crypts was observed with both low and high doses of Frondanol A5.

retrieval was carried out by heating sections in 0.01 mol/L citrate buffer (pH 6) for 30 min in a boiling water bath. Endogenous peroxidase activity was quenched by incubation in 3% H_2O_2 in PBS for 5 min. Nonspecific binding sites were blocked using Protein Block (normal serum) for 20 min. Sections were then incubated overnight at 4°C with 1:300 and 1:500 dilutions of rabbit monoclonal antibody against PCNA (Santa Cruz Biotechnology) and rabbit polyclonal antibody against p21 (Santa Cruz Biotechnology), respectively. After several washes with PBS, the slides were incubated with secondary antibody for PCNA and p21 for 2 h. The color reaction was developed by 3,3'-diaminobenzidine, according to the manufacturer's instructions given in the kit supplied by Zymed Laboratories. Substituted nonimmune rabbit immunoglobulins for primary antibodies were used as negative controls.

In vitro experiments

Reagents and antisera. Primary antibodies including anti-tubulin (1:1,000), anti-caspase-2 (1:300), anti-p21 (1:1,000), anti-PCNA (1:1,000), anti-Cdc25C (1:1,000), and anti-Cyclin B1 (1:500) were from Santa Cruz Biotechnology; anti-phospho histone H2ax (Ser139; 1:1,000) was from Assay Designs, Inc.; anti-phospho histone H3 (Ser10; 1:1,000) was from Cell Signaling; and fetal bovine serum, dimethyl sulphoxide, propidium iodide (PI), and Triton X were purchased from Sigma-Aldrich. McCoy's medium was purchased from Mediatech, Inc.

Cell culture and treatment. Human colon cancer HCT116 cells were purchased from the American Type Culture Collection, and maintained in McCoy's 5A medium containing L-glutamine and supplemented with 10% heat-inactivated fetal bovine serum, 100 U/mL penicillin, and 100 μ g/mL

streptomycin. Cells were incubated at 37°C in a humidified atmosphere with 5% CO₂ and subcultured after trypsinization (0.5% trypsin/2.6 mmol/L EDTA). For all experiments, cells were seeded at 1 × 10⁶ cells in culture dishes (100 mm) and grown to 60% to 70% confluence. At about 60% to 70% confluency, cultures were switched to serum-free medium for 24 h and then treated with different concentrations of Frondanol A5. Cells were treated with various concentrations of Frondanol A5 (dissolved in DMSO at <0.1%) for desired time periods, then the medium was aspirated. The cells were then washed twice with PBS and were processed as per the requirements of the experiments.

Cell proliferation assay. The CellTiter 96 AQueous One Solution Cell Proliferation Assay (Promega) was used to identify the number of viable cells in proliferation or cytotoxicity assays. HCT116 cells (5,000 cells) are layered in a 96-well plate for 24 h, and then treated with various concentrations of Frondanol A5 (10–150 µg) and incubated overnight at 37°C. After overnight incubation with the test compound, the CellTiter 96 AQueous One Solution Reagent was added to the cells. The solution reagent is bioreduced by active cells in 1 to 4 h into a colored formazan product, which is measured at 490 nm in a microplate reader (FLUOstar OPTIMA; BMG LABTECH). Data were derived from at least three independent experiments, and percentage of cell viability was calculated using the equation: [mean absorbance of treated cells/mean absorbance of control cells] × 100.

Nuclear morphologic changes. Morphologic changes in the nuclear chromatin of cells undergoing apoptosis were detected by the DNA-binding 4'-6-diamidino-2-phenylindole (DAPI, Sigma-Aldrich) stain. Briefly, HCT116 cells were grown on slides and exposed to Frondanol A5 (0 and 80 µg) for 24 h. Cells on the slides were fixed in 3% formalin for 30 min, washed thrice in PBS for 5 min, treated with DAPI in PBS containing 0.1% Triton X, and incubated for 10 min. The morphology of the cells' nuclei was observed using a fluorescence microscope (Olympus AX71) at excitation wavelength 350 nm connected to a digital imaging system with SPOT RT software version 3.0. Nuclei are considered to have the normal phenotype when glowing bright and homogeneously. Apoptotic nuclei were identified by the condensed chromatin gathering at the periphery of the nuclear membrane or a total fragmented morphology of nuclear bodies. The cells with apoptotic nuclei were photographed.

DNA ladder assay. The Frondanol A5-treated and untreated cells were washed in PBS, were resuspended in PureLink Genomic Digestion Buffer and 20 µL of Proteinase K (PureLink Genomic DNA kits), and were incubated at 55°C for 2 h until lysis was complete. Twenty microliters of RNase A were added to the lysate and incubated at room temperature for 2 min. PureLink Genomic Binding Buffer and 96% to 100% ethanol was added to the lysate. Further purification of DNA was carried out using spin columns as per the protocol instructions. The achieved DNA purity (A260/A280 = 1.8–1.9) was confirmed by spectrophotometry. DNA fragments were separated by 1.2% agarose gel electrophoresis, stained with ethidium bromide, and photographed under UV light.

Flow cytometric analysis for cell cycle arrest and apoptosis.

Flow cytometry and PI staining were used to determine the different phases of the cell cycle and percentage of apoptotic cells. After 24 h of test compound exposure, HCT116 cells were harvested, washed twice with PBS, centrifuged (~200 × g for 6 min), and fixed in 70% (v/v) ethanol at –4°C for 24 h. For cell cycle arrest, cells (10⁶–10⁷) were pelleted by centrifugation (~200 × g for 5 min), washed once with PBS, and resuspended in PI solution [20 µg/mL PI and 0.2 mg/mL RNase A in PBS (pH 7.4)] for 30 min at room temperature in the dark. For analysis of sub-G₁ phase, cells were harvested by trypsinization after treatment, washed in PBS, and incubated at 4°C for 16 h in the DNA staining solution containing 0.1% Triton X-100, 0.1% sodium citrate, and 50 µg/mL PI. Flow cytometric analysis was performed on a FACScan Flow Cytometer (Becton Dickinson). The red fluorescence of the single event was recorded using an argon ion laser at 488 nm excitation wavelength and 610 nm as emission wavelength to measure DNA index. The data from 50,000 cells were collected and analyzed using CellQuest Cell Cycle Analysis Software.

Protein expression by Western blot analysis. Cells exposed (24 h) to various concentrations of Frondanol A5 were harvested by gentle scraping and were lysed in ice-cold lysis buffer [50 mmol/L Tris (pH 7.4), 150 mmol/L NaCl, 1 mmol/L EDTA, 1% NP40, 50 mmol/L NaF, 1 mmol/L sodium orthovanadate, 1 mmol/L phenylmethylsulfonyl fluoride, 1 mmol/L DTT, and protease inhibitor cocktail]. After a brief vortexing, the lysates were clarified by centrifugation at 12,000 × g for 15 min at 4°C, and protein content was measured by the Bio-Rad Protein Assay reagent. An aliquot (50 µg protein/lane) of the total protein was separated by 10% or 15% SDS-PAGE and transferred to nitrocellulose membranes. After blocking in 5% milk, these membranes were tested for protein expressions of caspase-2, phospho histone H2AX, PCNA, Cdc25C, p21^(WAF1/CIP1), Cyclin B1, Phospho Histone H3 (ser10), and Tubulin (1:500, in TBS-Tween 20 solution) using respective primary antibodies, procured from Santa Cruz Biotechnology. The membranes and further probed with horseradish peroxidase-conjugated secondary antibody. Detection was done by using the SuperSignal West Pico Chemiluminescence procedure developed by Pierce. The bands captured on Ewen Parker, Blue sensitive X-ray films were analyzed by densitometry using image quant software. Immunoblotting with tubulin antibody was done to confirm equal protein loading.

Statistical analysis

The data are presented as mean ± SEM. Statistical differences between control and treated groups were evaluated using unpaired *t* test with Welch's correction. Differences between groups are considered significant at *P* < 0.05.

Results

General observation

All animals had a steady body weight gain during the treatment, and the administration of AOM and treatment

Table 1. Effects of dietary Frondanol A5 treatment on p21, and PCNA expression in colonic ACFs of azoxymethane-treated F344 rats

Experimental group	p21 expression (%)		PCNA expression (%)
	Nuclear	Cytoplasmic	
Control (azoxymethane + solvent) diet	20.1 ± 3.2*	19.2 ± 2.4	66.3 ± 4.6
Treated (azoxymethane + 450 ppm Frondanol A5) diet	59.1 ± 4.7 [†]	44.6 ± 3.5 [†]	28.6 ± 2.8 [†]

NOTE: PCNA and p21 expression was analyzed by immunohistochemistry using antibodies against PCNA and p21.

*Values are mean ± SEM ($n = 8-10$).

[†]Values are significantly different from control group, by two-tailed Student's *t* test with Welch's correction.

with 0, 150, or 450 ppm Frondanol A5 did not affect the growth of the rats as measured at weekly intervals. Diet consumption of groups fed 150 or 450 ppm was not different from that of the azoxymethane-treated positive control group or rats fed 160 ppm of sulindac diet. During the entire period of experiment, there were no signs of toxicity or conditions suggesting adverse effects caused by dietary administration of Frondanol A5.

Dietary Frondanol A5 treatment inhibited colonic ACF formation

Vehicle-treated rats fed with modified AIN to 76A or experimental diets containing Frondanol A5 showed no evidence of ACF formation in the colon (data not shown). The efficacy end points used in this study were inhibition of total ACF/colon and reduction of number of multicrypt clusters (four or more) of aberrant crypts/colon. Azoxymethane-treated rats that were fed with the control diet induced, on the average, ~146 ACF/colon and 35 foci/colon containing multiple (4 or more) aberrant crypts/focus (Fig. 1B and C). As expected, ACFs were predominantly observed in the distal colon. Frondanol A5 diet-fed rats showed significant inhibition of total occurrences of ACF/colon (34-55%, $P < 0.0001$) and of multicrypt clusters containing three, or four, or more crypts/focus (48-70%, $P < 0.0001$; Fig. 1B and C). Sulindac, a well-established chemopreventive agent and known inhibitor of colon carcinogenesis in animal assays, reduced polyps in patients with familial polyposis, and used for comparison study, was found to be an effective inhibitor of total occurrence of ACF/colon (50%, $P < 0.0001$) and of multicrypt clusters containing four or more crypts/focus (56.1%, $P < 0.0001$; Fig. 1B and C). In the present study, Frondanol A5 reduced azoxymethane-induced colonic total ACFs and multicrypt ACFs in a dose-dependent manner.

Inhibition of PCNA expression in high-grade dysplastic ACF by Frondanol A5

Qualitative microscopic examination of PCNA-stained sections showed a substantial decrease in PCNA-positive cells in the ACFs from Frondanol A5-treated rats compared with untreated controls. The quantification of PCNA staining showed 28.6 ± 2.8% (mean ± SEM) PCNA-positive cells

in ACF of rats fed Frondanol A5 (450 ppm), compared with 66.3 ± 4.6% (mean ± SEM) PCNA-positive cells in azoxymethane-treated control diet rats, accounting for a decrease in proliferation by ~46% ($P < 0.0001$; Table 1 and Fig. 2A).

Restoration of p21 expression in azoxymethane-induced ACFs by Frondanol A5

ACFs with high-grade dysplasia from positive control and 450 ppm Frondanol A5-treated rats were analyzed for p21 induction by immunohistochemistry (Fig. 2B). p21-stained positive cells displaying nuclear and cytoplasmic staining were observed in normal mucosa, but to a relatively lesser extent in the ACFs of control diet-fed rats. Frondanol A5 treatment did cause an appreciable increase in the p21-positive cells in colonic ACFs. Using the staining intensity of p21 in normal epithelial cells as the internal standard, immunohistochemistry evaluation showed that all observed high-grade dysplastic ACF cells from the untreated control group showed 20.1 ± 3.2% (mean ± SEM) nuclear p21 staining. In the dietary Frondanol A5-treated group, positively stained cells in ACF showed a 66.1% increase compared with the untreated control group ($P < 0.0001$; Table 1 and Fig. 2A).

Frondanol A5 decreases HCT116 cell viability

As shown in Fig. 2C, Frondanol A5 decreased HCT-116 cell viability in a time- and dose-dependent manner with a half maximal inhibitory concentration (IC_{50}) of approximately 65 ± 3.7 µg/mL after 24 hours. It is noteworthy that even at concentrations >150 µg/mL, Frondanol A5 was not fully cytotoxic and these *in vitro* experiments were carried using concentrations of <150 µg/mL.

Induction of apoptosis

We observed that Frondanol A5 affected the HCT-116 cell morphology as treated cells became rounded, detached from the culture plate, and showed membrane blebbing (Fig. 2D), suggesting that induction of the apoptotic pathway did occur. To test this hypothesis, we performed DAPI staining, DNA fragmentation (DNA ladder assay), and flow cytometric analysis. Almost all untreated cells maintained a high nucleocytoplasmic ratio with a large and round-shaped nucleus. The typical apoptotic features, such as unfragmented or irregularly fragmented

pyknotic nuclei, were observed in treated cells upon DAPI staining (Fig. 2E). Further, agarose gel electrophoresis of DNA extract from treated and untreated cells to analyze fragmented nuclei has confirmed DNA fragmentation (a hallmark of apoptosis). Frondanol A5 caused profound internucleosomal DNA fragmentation, which was shown by a prominent DNA fragmentation ladder assay on agarose gel electrophoresis. Cleaved DNA fragments in multiples of $\sim 180/200$ -bp oligomers were visualized as a DNA ladder on agarose gel, which is a characteristic feature of apoptotic cell death that distinguishes it from other forms of cell death (Fig. 2F). Additional evidence from flow cytometric analysis revealed a

substantial dose-dependent increase in the hypodiploid (sub-G₁) peak, which represents the fraction of fragmented apoptotic cells (Fig. 2G).

Induction of caspase-2

To study the possible involvement of caspase activation in Frondanol-induced apoptosis, we examined the cleavage of caspase-2 by Western blotting. The effect of Frondanol A5 on caspase activation was investigated on early treatment time points (30 minutes to 8 hours), which showed the activation of caspase-2 as early as 1 hour of treatment (Fig. 3A). As shown in Fig. 3A, we found that Frondanol A5 at 65 $\mu\text{g/mL}$ induced caspase-2

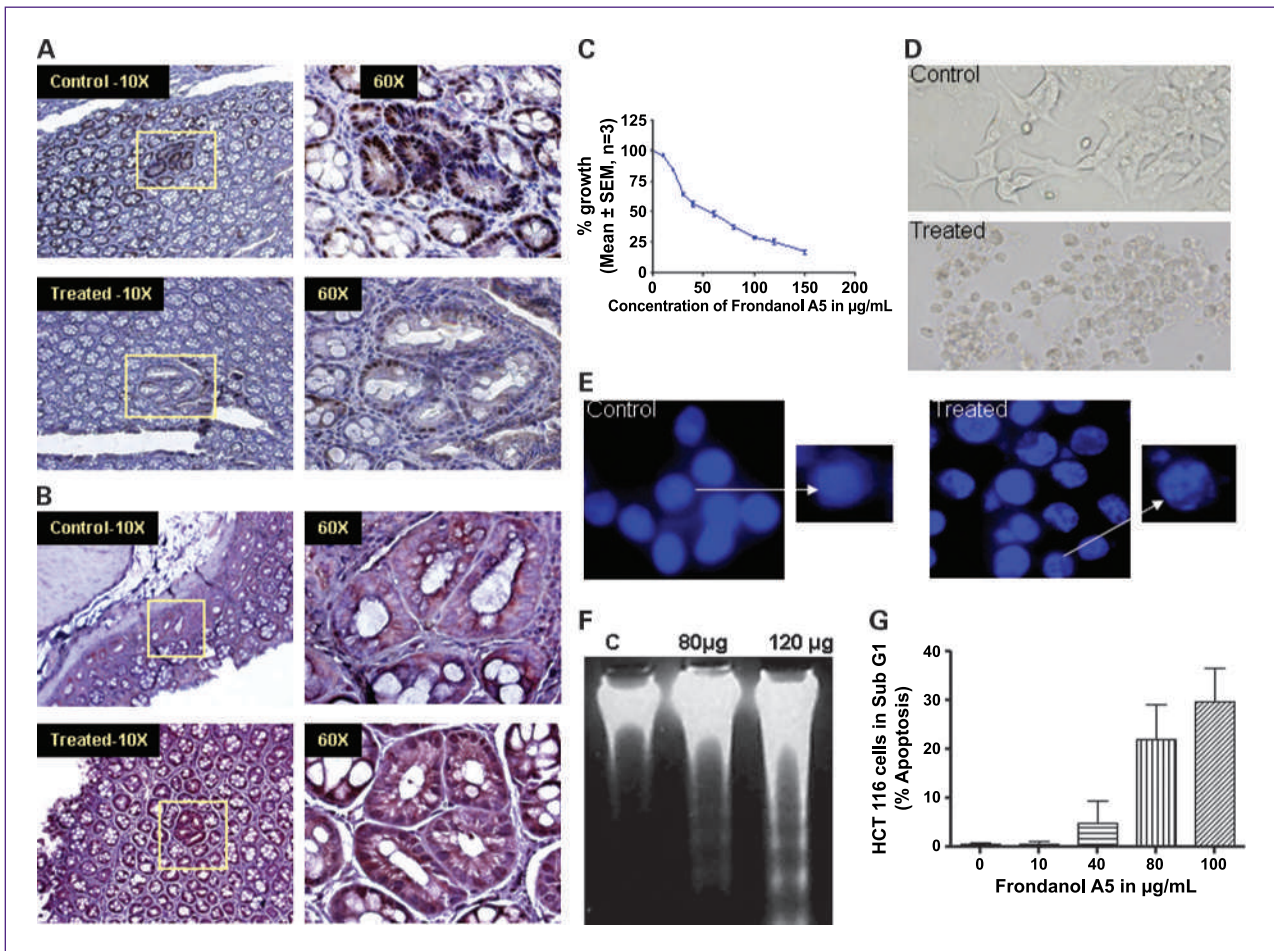


Fig. 2. A, serial paraffin sections of a colon ACF induced by azoxymethane were subjected to immunohistochemical analysis using anti-PCNA monoclonal antibody. The intense positive staining of PCNA in the ACF region of control animals was observed with a decrease of PCNA in the nucleus of dysplastic ACF of animals treated with 450 ppm of Frondanol A5. An enlarged image of the accumulation of PCNA is shown. B, increase of p21 in the nucleus of dysplastic ACF. B, Serial paraffin sections of dysplastic ACF induced by azoxymethane were subjected to immunohistochemical analysis using an anti-p21 polyclonal antibody. Marked accumulation of p21 is clear in the nucleus of dysplastic ACF in treated animals compared with control animal ACF; nuclear accumulations of p21 are visualized by enlarged image. Magnifications are $\times 10$ and $\times 60$. C, Frondanol decreases HCT116 cell viability in a dose-dependent manner. Evaluation of HCT116 cell viability after 24 h of treatment with Frondanol A5 (0-150 $\mu\text{g/mL}$), determined by MTS assay as described previously in Materials and Methods. Points, mean of three independent experiments; bars, SEM. D, light microscope examination of HCT116 cell morphology after 24 h of treatment with 0 and 80 $\mu\text{g/mL}$ of Frondanol A5. E, fluorescence microscopy analysis of HCT116 cells treated with 80 $\mu\text{g/mL}$ Frondanol A5 and stained with 0.5 $\mu\text{g/mL}$ DAPI. F, Frondanol A5 induces apoptosis, analyzed by DNA ladder assay as described in Materials and Methods. G, flow cytometric analysis of sub-G₁ phase DNA content in HCT116 cells after 24 h of incubation with 0 to 100 $\mu\text{g/mL}$ Frondanol A5 and stained with 50 $\mu\text{g/mL}$ PI, as described previously in Materials and Methods.

activation, starting from 30 minutes of treatment onwards, a time-dependent cleavage of the procaspase-2 into its active form. An induction of cleavage of procaspase-2 was observed at 6 to 8 hours. Frondanol A5 showed a slight increase in cleaved caspase-2 (18, 14, 12 kDa) at 2 hours, which became prominent at 8 hours of Frondanol A5 treatment (Fig. 3A). These results agree with H2AX phosphorylation, a known marker of DNA double-strand breaks (Fig. 3A).

Frondanol A5 induces double-strand breaks and phosphorylates H2AX at Ser 136

It is proposed that DNA double-strand breaks are common ultimate apoptosis-triggering lesions arising from primary DNA lesions during DNA replication. We analyzed the H2AX phosphorylation in HCT-116 cells after treatment with Frondanol A5. Western blot analysis of treated and untreated cells revealed a dose-dependent and time-dependent phosphorylation of H2AX and caspase-2 (Fig. 3A-C). H2AX phosphorylation followed a similar time course as that of caspase-2, suggesting a parallel response (Fig. 3A and B). Recent studies from various laboratories have established a role for caspase-2 during genotoxic stress-induced apoptosis (15, 16). Studies showed that caspase-2 is activated early in response to DNA damage acting upstream of mitochondria, suggesting that it functions as an apical caspase (15, 17–20). We

also observed that Frondanol A5 treatment (10–120 $\mu\text{g}/\text{mL}$) suppresses the PCNA expression in a dose-dependent manner in HCT116 cells (Fig. 3D).

H2AX phosphorylation is associated with the G₂-phase checkpoint

In cells treated with Frondanol A5 (65 $\mu\text{g}/\text{mL}$) for 6 hours, γ -H2AX formation increased in a dose-response manner (Fig. 3A and C). γ -H2AX formation is considered to be a sensitive and selective signal for the existence of double-strand break (21, 22). It is possible that phosphorylation of H2AX is an indication of the early DNA damage sensor response that culminates in the activation of S-phase arrest and more of G₂-M-phase arrest. We investigated if γ -H2AX is associated with the G₂-phase checkpoint by analyzing the protein markers that control cell cycle regulation. Several recent studies suggest that p21^{Waf1/Cip1} regulate the entry of cells at DNA damage-induced G₂-M checkpoint and induce apoptosis (23–25). Western blot analysis revealed that the treatment of cells with 40 $\mu\text{g}/\text{mL}$ of Frondanol A5 for 24 hours resulted in a marked induction of the protein expression of p21^{Waf1/Cip1} (up to 6-fold higher at higher dose levels) and cyclin B1 in a dose-dependent manner compared with untreated cells (Fig. 3E). The protein expression of Cdc25C was drastically reduced following the treatment of cells with

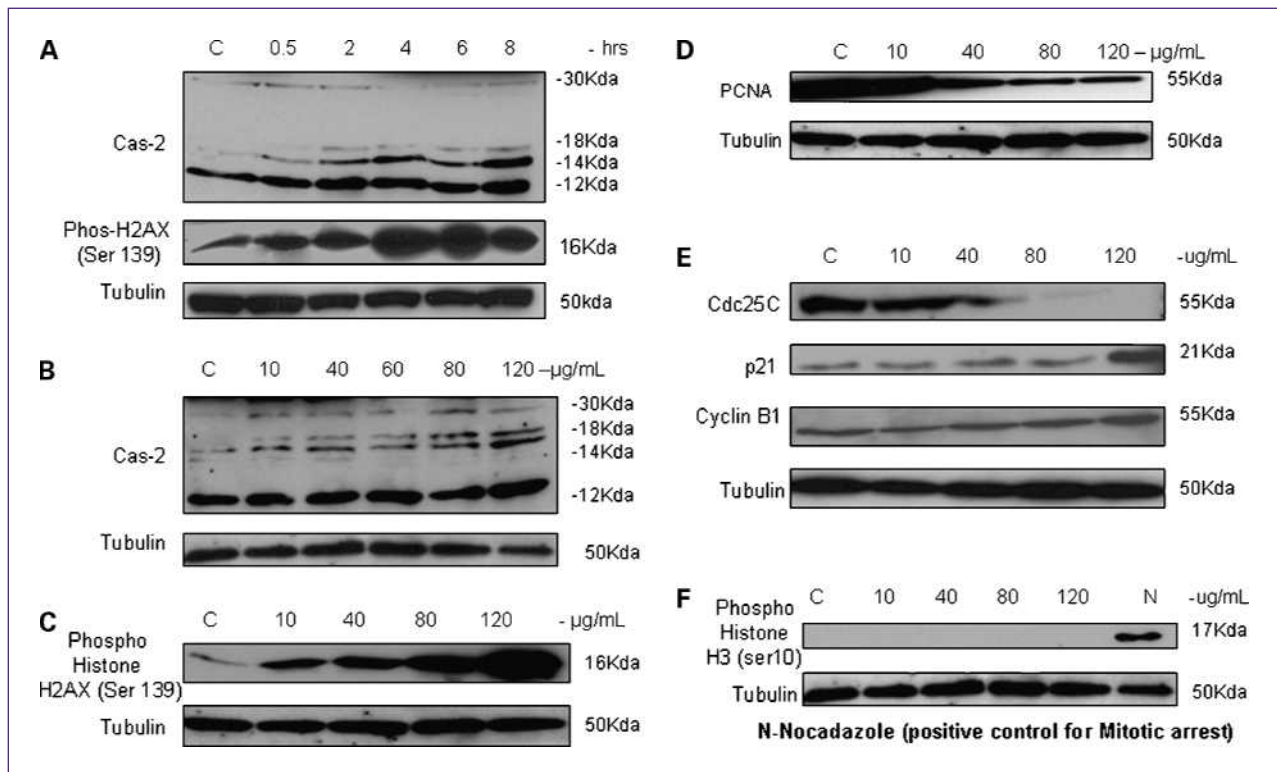


Fig. 3. A, effect of Frondanol A5 on HCT116 cells at different time intervals (30 min to 8 h) on caspase-2 (Cas-2) and Phos-H2AX. B, dose-dependent effect of Frondanol A5 on HCT116 cells on caspase-2. C, the effect of Frondanol A5 on DNA damage marker. D, Western immunoblot to identify the effect of Frondanol A5 on proliferation marker (PCNA). E, Western immunoblot to identify the mitotic block on HCT116. F, Western immunoblot to identify the effect of Frondanol A5 on cell cycle markers in HCT116.

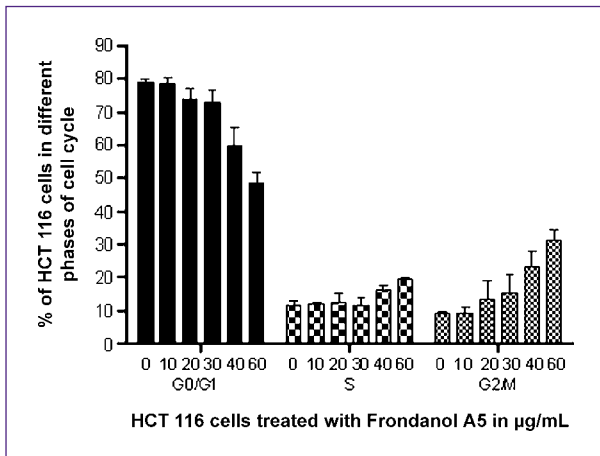


Fig. 4. Flow cytometric analysis of DNA content in HCT116 cells present in different phases of cell cycle, after 24 h incubation with 0 to 60 µg/mL of Frondanol A5, as described in Materials and Methods.

Frondanol A5 for 24 hours from 40 µg/mL upwards (~60% reduction relative to the control), indicating its role in Frondanol A5-mediated G₂-M arrest (Fig. 3E). These results are consistent with an action of the G₂-M phase checkpoint, 24 hours after Frondanol A5 treatment (Fig. 3F). Further, we determined the effect of Frondanol A5 treatment on the protein expression and phosphorylation of histone H3 at Ser-10, a mitotic marker. There was no phosphorylation and protein expression of histone H3 observed following treatment of cells with Frondanol A5 for 24 hours compared with positive control, nocadazole-treated cells (Fig. 3F). This indicates that Frondanol A5 is mediating G₂ arrest and not mitotic arrest (Fig. 4).

Discussion

Administration of Frondanol A5, a sea cucumber glycolipid extract, decreased azoxymethane-induced colonic ACF formation in F344 male rats. ACFs are one of the earliest lesions of colon carcinogenesis (14, 26, 27) and are regarded as putative preneoplastic conditions and may serve as intermediate markers for colon cancer in rodent models and in human. Some of the molecular changes described in human colon cancers are also associated with colonic ACF, such as K-ras, adenomatous polyposis coli, and β-catenin mutations, and growth-promoting alterations in cell cycle-controlling genes (28–30). Thus, findings in experimental animal models and studies in humans strongly support the premalignant potential of ACF. The potency of over 60 chemopreventive agents inhibiting rat ACF correlates extremely well with their effects on rat tumor formation (31). In the present study, we used sulindac, a positive inhibitor of colonic ACF. As expected, sulindac was found to be a strong inhibitor of azoxymethane-induced colonic ACF (Fig. 1B and C; ref. 32). This is the first study to show that dietary administration of Frondanol A5 suppresses chemically induced colon car-

cinogenesis. Our earlier studies have shown that triterpenoids up to 1,500 ppm in diet as chronic feeding does not show any toxicity, and most of our ACF studies are carried out with much less than 750-ppm doses. Thus, in the present study, 150 and 450 ppm of Frondanol A5 were used based on the previous *in vivo* studies and recent organ culture experiments. In this study, dietary administration of 150 and 450 ppm of Frondanol A5 significantly suppressed azoxymethane-induced total colonic ACF and multicrypt (four or more) aberrant foci formation in a dose-dependent manner. Furthermore, Frondanol A5 suppressed azoxymethane-induced colonic ACF was associated with a decrease in the proliferation marker PCNA and an increase in p21 expression levels. These results suggest that Frondanol A5, at least in part, suppresses colonic ACF formation by suppressing the colonic epithelial cell proliferation.

Frondanol A5-induced anticancer properties are not limited to colon cancer. Previous studies with this agent have shown the suppression of pancreatic and breast cancer cell growth (33). In addition, Frondanol A5 has been shown to inhibit 86.4% of B[a]P-induced rat tracheal epithelial foci transformation (9). Dietary supplementation of Frondanol A5 in combination with other agents significantly enhanced the anti-inflammatory activities of other agents (13). One of the major advantages of Frondanol A5 is that unlike synthetic chemopreventive agents, it is a naturally occurring compound present in the edible sea cucumber with no toxicities documented. The exact mechanism(s) involved in the inhibitory effect of azoxymethane-induced colon carcinogenesis by dietary Frondanol A5 is not yet fully known. Previous studies have shown that Frondanol A5 might inhibit inflammatory pathways; however, our *in vitro* and *in vivo* data support proliferation inhibition by upregulation of cell cycle regulators and induction of apoptosis by caspase.

Enhanced crypt cell proliferation in dysplastic ACF supports the significance of crypt cell hyperproliferation as a biomarker of ACF with greater neoplastic potential (34). Increase in PCNA staining in ACF leads to higher risk for malignant progression (35, 36). PCNA, which is widely used as a marker of cell proliferation, was assessed by immunohistochemistry in ACFs of Frondanol A5-treated versus untreated animals. A significant decreased staining of PCNA in ACFs of treated rats suggests antiproliferative properties of sea cucumber extract Frondanol A5 (Fig. 2A). Upregulation of p21 with decreased PCNA in ACFs of treated rats further supports the potential of Frondanol A5 for colon cancer chemoprevention (Fig. 2B). Previous studies have shown that increased tumor formation is associated with altered cell maturation in the intestinal mucosa of the p21-deficient mice, which harbors increased cell proliferation, decreased apoptosis, and goblet cell differentiation (37).

Caspase-2 is a direct effector of the mitochondrial apoptotic pathway (19) and possesses an unparalleled ability to engage the mitochondrial apoptotic pathway by

permeabilizing the outer mitochondrial membrane and/or by breaching the association of cytochrome *c* with the inner mitochondrial membrane (15). In this study, we showed that caspase-2 was activated during apoptosis induced by Frondanol A5 treatment. Although the exact mechanism of caspase-2 activation is not yet clear, it has recently been found that caspase-2 can be activated in different cells by various stimuli, causing DNA damage (38, 39). Interestingly, in accordance with these reports, we have found that Frondanol A5 induces time-dependent DNA damage along with the activation of caspase-2 in HCT116 colon cancer cells (Fig. 3A and B). Phosphorylated form of H2AX, called γ -H2AX, appears during apoptosis concurrently with the initial appearance of high molecular weight DNA fragments. γ -H2AX formation is an early chromatin modification following initiation of DNA fragmentation during apoptosis (40). Frondanol A5 caused morphologic changes characteristic of apoptosis in the HCT116 cells, such as progressive chromatin condensation along with γ -H2AX phosphorylation (Figs. 2D and 3C).

On the other hand, we have found a transient accumulation of the treated HCT116 cells at the S and G₂ phase of the cell cycle, triggering apoptosis. Previous studies have shown that cell cycle arrest may occur as a result of DNA damage that makes the cells arrested at the G₂-M checkpoint to allow DNA repair and to prevent entry into mitosis in the presence of damaged DNA (41–43). Arita et al. (41) have shown in colon cancer cell lines that apoptotic induction by DNA damage is not necessarily related to p53 status and that induction of p53-independent apoptosis following DNA damage may correlate with G₂ arrest in the cell cycle. We have not observed any changes in the p53 expression on Frondanol A5 treatment in *in vitro* assays. Indeed, the changes in cell cycle observed in this study (upregulation of cyclin B1 and upregulation of p21) are typical to the expected changes that are achieved when cells block in G₂ phase. In summary, Frondanol A5 inhibits cell growth by inducing a G₂ arrest followed by induction of apoptosis (Figs. 3F and 4). Increased p21 levels and upregulation of cyclin B1 expression may be a possible underlying mechanism for this G₂ arrest. In this study, flow cytometric analysis clearly revealed that human colon carcinoma HCT-116 cells were arrested by Frondanol A5 at the G₂-M phase of the cell cycle, which was further confirmed that the block is at the G₂ phase by using a mitotic marker [phosphorylated histone H3 formation (ser10); Figs. 3F and 4]. Frondanol A5

induced intracellular protein levels of cyclin B1 in HCT116 cells in a dose-dependent manner (Fig. 3E). Of several cyclin-dependent kinase inhibitors, p21, an inhibitor of Cdk2, is an important mediator of cell cycle arrest in response to DNA damage (18). It was shown that a novel form of p21 protein can inhibit growth by acting not at G₁ but at G₂-M (44, 45). In addition to being induced by p53, p21 is also induced by other factors independent of p53 (15, 46). The present results clearly indicated that Frondanol A5 enhanced the expression of cyclin-dependent kinase inhibitor p21, without altering the p53 expression (Fig. 3E).

In conclusion, dietary administration of Frondanol A5 isolated from sea cucumber reduced the formation of colonic ACF, inhibited expression of cell proliferation and cell signaling biomarkers, and induced apoptosis. Development of dietary agents is particularly attractive because of our long-standing exposure to them, their relative lack of toxicity, and their etiologic significance. Thus, results from this study for the first time suggest that the sea cucumber extract, Frondanol A5, might be further developed as a possible chemopreventive agent for colon cancer.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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