β-Ionone inhibits colonic aberrant crypt foci formation in rats, suppresses cell growth, and induces retinoid X receptor-α in human colon cancer cells

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Abstract

β-Ionone, an end-ring analogue of β-carotenoid, which is a constituent of vegetables and fruits, has been analyzed for colon cancer chemoprevention and treatment. β-Ionone induced cell growth inhibition and apoptosis in human colon cancer HCT116 cell line. We tested the *in vivo* chemopreventive efficacy in rat colon carcinogenesis model using aberrant crypt foci (ACF) as endpoint marker. HCT116 cells treated with subtoxic concentrations of β-ionone resulted dose-dependent cell growth suppression with G_1-S-phase growth arrest and significant induction of apoptosis. β-Ionone up-regulated expression of retinoid X receptor-α mRNA dose-dependently in HCT116 cells. To evaluate inhibitory properties of β-ionone on colonic ACF, 7-week-old male F344 rats were fed experimental diets containing 0%, 0.1%, or 0.2% β-ionone. After 1 week, rats received s.c. injections of azoxymethane, 15 mg/kg body weight, once weekly for 2 weeks. Rats were continued on respective experimental diets and sacrificed 8 weeks after the azoxymethane treatment. Colons were evaluated histopathologically for ACF. Administration of dietary 0.1% and 0.2% β-ionone significantly suppressed total colonic ACF formation up to 34% to 38% ($P < 0.0002$ to $P < 0.0009$), respectively, when compared with control group. Importantly, rats fed β-ionone showed >55% inhibition ($P < 0.0001$) of foci containing four or more aberrant crypts. Results from *in vitro* and *in vivo* bioassay clearly suggest that β-ionone could be further developed for prevention and treatment of colon cancer. [Mol Cancer Ther 2008;7(1):181–90]

Introduction

International dietary guidelines for the prevention of chronic diseases recommend increased consumption of plant foods, including fruits, vegetables, grains, etc. Such plant foods contain the traditional macronutrients and a wide variety of physiologically active phytochemicals (1). Phytochemicals and micronutrients present in fruits and vegetables are known to exert cancer chemopreventive effects in several organs, including the colon. To date, several reports have described that intake of these constituents could lead to a reduced risk of chronic diseases, such as cancer and cardiovascular disorders (2–6). β-Ionone is one such phytochemical present in many fruits, vegetables, and grains and is a common intermediate product of up to 22,000 isoprenoids, including carotenoids (7). They are important dietary sources of vitamin A (8).

β-Ionone is a precursor for carotenoids and two molecules of β-ionone combine to form β-carotene. Products of enzymatic degradation of carotenoids are found to be ionones. β-Ionone chemical structure is somewhat similar to retinol that, in part, determines its physiochemical properties and biological activities (Fig. 1). Retinoic acids (which are generated in intestine from carotenoids) and their corresponding nuclear receptors, the retinoic acid receptors α, β, and γ and the retinoid X receptors (RXR) α, β and γ, regulate the cell growth, differentiation, and apoptosis (9, 10). Nuclear receptors function as ligand-activated transcription factors that regulate the expression of target genes to affect almost all biological processes as diverse as reproduction, development, and general metabolism (11). RXRs are a family of nuclear receptors implicated in cancer chemoprevention.

β-Ionone is found to exert anticarcinogenic and antitumor activities in melanoma, meningioma cells, and breast cancer (12–17). It is also reported that β-ionone can induce apoptosis in tumor cells (18–19). To our knowledge, no data exist on the possible colon tumor growth inhibitory effects of β-ionone and its proliferative mechanisms. In the present study, we studied growth inhibitory, apoptosis induction, and modulation of RXR in human colon cancer HCT116 cell line. We also examine the chemopreventive efficacy of β-ionone on azoxymethane-induced rat colon carcinogenesis model using colonic aberrant crypt foci (ACF) as endpoint biomarker.

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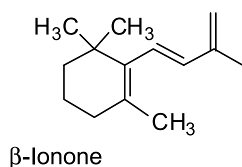

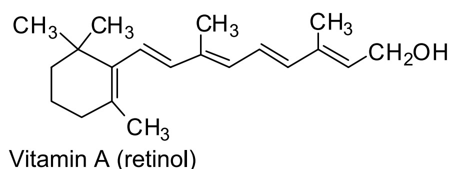

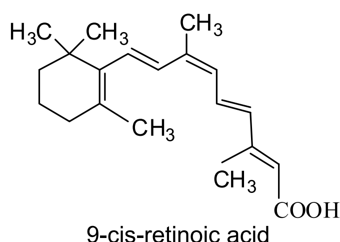

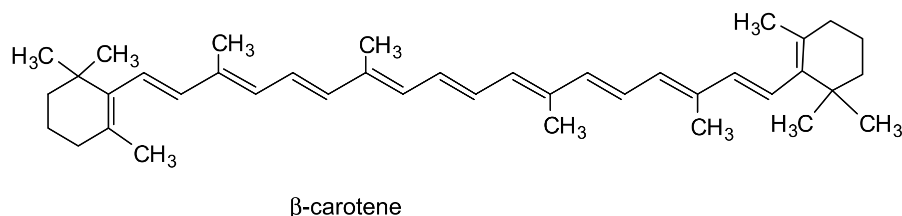

Figure 1. Structures of β-ionone, β-carotene, retinol, and 9-*cis*-retinoic acid. Structurally, β-ionone is similar to ring structures of β-carotene, retinol, and 9-*cis*-retinoic acid. These molecules posses a β-ionone ring in their structure, which interacts with respective retinoic acid receptor/RXRs.

Materials and Methods

Cell Culture

Human colon cancer HCT116 cells were purchased from American Type Culture Collection and maintained in McCoy's 5A medium containing L-glutamine and supplemented with 10% heat-inactivated fetal bovine serum, 100 units/mL penicillin, and 100 μg/mL streptomycin. Cells were incubated at 37°C in a humidified atmosphere with 5% CO_2 and subcultured after trypsinization (0.5% trypsin/2.6 mmol/L EDTA). For all experiments, cells were seeded at 1×10^6 in culture dishes (100 mm) and grown to 60% to 70% confluence. To study the growth arrest, apoptosis, and RXR-α mRNA expression, we used various subtoxic doses of β-ionone ranging from 20 to 100 μmol/L.

Cell Proliferation Assay

Ubiquitous lysosomal enzyme *N*-acetyl-β-D-hexosaminidase, an endogenous enzyme, is used to measure the viable cell numbers by using substrate *p*-nitrophenol- *N*-acetyl-β-D-glucosaminide (20). HCT116 cells (n = 5,000) are layered in a 96-well plate for 24 h, treated with various concentrations of β-ionone (10-250 μmol/L), and then incubated overnight at 37°C. After overnight incubation, enzyme substrate is added and allowed to react with the endogenous enzyme. Enzyme activity is measured at 405 nm in 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.

Detection of Apoptosis

Cells were exposed to β-ionone (25, 50, 100, 150, and 200 μmol/L) for 48 h. The cell suspension (25 μL; ~5×10^6/mL) was added with 1 μL acridine orange/ethidium bromide (one part each of 100 μg/mL acridine orange and 100 μg/mL ethidium bromide in PBS) just before microscopy. A 10 μL aliquot of the gently mixed suspension was placed on microscope slides, covered with glass slips, and examined under an Olympus AX71 microscope connected to a digital imaging system with SPOT RT software version 3.0. Live cells stain uniformly green and can be distinguished from apoptotic cells, which exhibit yellowish/

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orange dots of condensed chromatin. Apoptotic cells that have lost their membrane integrity appear orange due to costaining with ethidium bromide showing condensed chromatin. Cells were scored into following categories: C1, cells with large, green, noncondensed nuclei as nonapoptotic, viable cells; C2, cells with red/orange nuclei that showed signs of nuclear bead formation as apoptotic cells; and C3, cells with large red nuclei that did not show signs of nuclear condensation or bead formation as necrotic cells. At least 200 cells per sample were counted and scored. The apoptotic index (%) was calculated by dividing the sum of apoptotic cells C2 × 100 by the total number of cells scored.

Cell Cycle Arrest by Flow Cytometry

Flow cytometry and propidium iodide staining were used to determine the different phases of the cell cycle as described (21). After 24 h of β-ionone exposure, HCT116 cells were harvested, washed once with PBS, centrifuged (~200 × *g* for 6 min), and fixed in 70% (v/v) ethanol at -4°C for 24 h. Cells (10^6-10^7) were pelleted by centrifugation (~200 × *g* for 5 min), washed once with PBS, and resuspended in propidium iodide solution [20 μg/mL propidium iodide and 0.2 mg/mL RNase A in PBS (pH 7.4)] for 30 min at room temperature in the dark. 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. Flow cytometric analysis was done on a FACScan flow cytometer (Becton Dickinson). The data from 50,000 cells were collected and analyzed using CellQuest Cell Cycle Analysis Software.

Expression of RXR-α mRNA Using Real-time PCR

The real-time PCR was carried out in a 25 μL reaction volume using 3 μL of a 1:10 cDNA dilution containing HotStart Taq DNA polymerase (Sigma), iQ SYBR (Molecular Probes), 10× PCR buffer (containing 3.5 mmol/L Mg), deoxynucleotide triphosphates 200 μmol/L of each and primers (300 nmol/L), forward primer (5-AACCCCTGA-AAGCAGAACCT-3) and reverse primer (5-TACAAGGCC-GATGAGAAAGG-3), which allowed amplification of a 110-bp nucleotide fragment of RXR-α mRNA for HCT116 cells. Forward and reverse primers for rat RXR-α mRNA are 5-TCCGAGTAGGGACCAAGAGA-3 and 5-GCTT-TACTGCAGCTGCCTCT-3, respectively, which amplified a 102-bp nucleotide fragment. As an internal control, a 100-bp fragment of β-actin using primers, forward primer 5-ATCATTGCTCCTCCTGAGCG-3 and reverse primer 5-GCTGATCCACATCTGCTGGAA-3, was also amplified. Both amplifications were carried out in triplicate for each reverse transcription product. No-template control was also included. The cDNA samples were then amplified at 95°C for 3 min, 95°C for 10 s, and 55°C for 30 s for a total of 40 cycles. All PCRs were done in an iCycler iQ real-time PCR detection system. The fluorescence threshold values (Ct) were calculated using the manufacturer's software. Relative mRNA levels were assessed by standardization β-actin. Results were expressed as a *n*-fold difference in gene expression.

In vivo Experiments

Animas, Diet, and Care. All animal experiments were done in accordance with the institutional guidelines of the American Council on Animal Care. Male F344 rats were obtained from Charles River Laboratories, housed under standardized conditions (21°C, 60% relative humidity, 12-h light/12-h dark cycle, 20 air changes per hour), and fed a standard laboratory rodent chow and drinking water until initiation of the experiment. Diets were prepared based on modified AIN-76A containing 5% corn oil by weight (American Institute of Nutrition). β-Ionone (>96% pure) was purchased from Sigma Chemical. The experimental diets contained 0.1% (1,000 ppm) or 0.2% (2,000 ppm) of β-ionone. Diets were prepared once each week and stored at 4°C until used. Rats were allowed *ad libitum* access to the respective diets and tap water.

Determination of the Maximum Tolerated Dose of β-Ionone. The purpose of this maximum tolerated dose study was to determine the tolerable dose of β-ionone in F344 rats. Maximum tolerated dose is defined as the highest dose that causes no more than 10% weight decrement, compared with the appropriate control diet group, and does not produce mortality or any clinical signs of toxicity that would be predicted to shorten the natural lifespan of the animal. At 6 weeks of age, groups of male F344 rats (six rats per group) were fed the AIN-76A diet containing 0%, 0.04%, 0.08%, 0.12%, 0.16%, and 0.2% β-ionone. Body weights were recorded once weekly for 6 weeks. All animals were killed after 6 weeks and the organs were examined grossly for any abnormalities.

Experimental Design for Efficacy of β-Ionone. The experiment was designed to evaluate the efficacy of 0.1% and 0.2% β-ionone administered continuously from 1 week before carcinogen treatment to the end of the study. The dose selection was based on our maximum tolerated dose study. At 7 weeks of age, groups of rats (*n* = 18 rats per group; 12 azoxymethane-treated rats plus 6 vehicle-treated rats) were fed either the control diet or the experimental diet containing 0, 0.1%, or 0.2% β-ionone. At 8 weeks of age, rats intended for carcinogen treatment were injected s.c. with azoxymethane (Midwest Research Institute) at a dose rate of 15 mg/kg body weight once weekly for 2 weeks, and those intended for vehicle treatment received an equal volume of normal saline. These dietary regimens were continued until termination of the experiment (i.e., 8 weeks after the second azoxymethane treatment). Rats were killed by CO_2 euthanasia and all organs were examined grossly. Colons were evaluated for ACF. For this evaluation, they 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.

Quantification of ACF. Topographic analysis of the colonic mucosa according to Bird (22) was done after a minimum of 24-h fixation. Colons were stained with 0.2% methylene blue solution for 5 to 10 min, placed mucosal side up on a microscopic slide, and viewed under a light microscope. The total number of ACF in the entire colon was determined in every 2 cm section of the colon, starting

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from the distal (taken as 0 cm) to the proximal end of the colons. Aberrant crypts were distinguished from the surrounding normal crypts by their increased size, increased distance from lamina to basal surfaces of cells, and easily discernible pericryptal zone. The variables 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 up to four or more aberrant crypts/focus.

Induction of Colon Tumors and Immunohistochemistry. To assess the expression levels of RXR-α in colon tumors and normally appearing mucosa, rats were fed control AIN-76A diet and treated with azoxymethane (15 mg/kg body weight once weekly for 2 weeks). Colonic tumors and normal tissues were harvested 40 weeks after the azoxymethane treatment.

Rat colon tumor and normal tissue sections (obtained by azoxymethane treatment) were dried at 56°C, deparaffinized in xylene, rehydrated, and washed with PBS for 15 min at room temperature. Specimens were treated in water bath in 0.01 mol/L citrate buffer (pH 6.0) for 30 min at 100°C, slowly cooled to room temperature, and washed with PBS for 5 min at room temperature. After quenching endogenous peroxide with 3% hydrogen peroxide in PBS for 10 min at room temperature, the sections were incubated with a blocking solution (supplied by Zymed Laboratories kit) for 60 min at room temperature. Then, the slides were incubated overnight at 4°C with a 1:300 dilution of anti-RXRα (Santa Cruz Biotechnology). After several washes with PBS, the slides were incubated with secondary antibody for RXR-α 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.

Statistical Analysis

Data are reported as mean ± SE. 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

Effect of β-Ionone on HCT116 Cell Growth and Death

HCT116 cells were exposed for 24 h to β-ionone at concentrations varying from 0 to 250 μmol/L. As shown in Fig. 2*A*, cell growth inhibition was observed in a dose-dependent manner. At ~60 μmol/L, β-ionone induced 50% inhibition of cell growth after 24 h of exposure. At 200 μmol/L or above concentrations, β-ionone induced significant toxicity in HCT116 cells. All the *in vitro* experiments with β-ionone were done with <60 μmol/L concentration, except for apoptosis assays, which was checked up to 200 μmol/L to observe morphological changes at higher concentrations.

β-Ionone Induced Apoptosis in HCT116 Cells

We observed the apoptotic bodies by staining with ethidium bromide and acridine orange. The population of apoptotic cells was significantly ($P < 0.0002$) enhanced in β-ionone-treated cells. As shown in Fig. 2*B* and *C*, β-ionone induced apoptosis in HCT116 cells in a dose-dependent manner (5.6% in 25 μmol/L, 10% in 50 μmol/L, 21% in 100 μmol/L, and 79% in 150 μmol/L) versus untreated cells.

β-Ionone Suppresses Proliferating Cells at G_1 Phase of the Cell Cycle

We examined the effect of β-ionone on HCT116 cell cycle by flow cytometry. Colon cancer cells were exposed to increasing concentrations of β-ionone for 24 h and subsequently processed for cell cycle analysis. Results are summarized in Table 1. We observed that asynchronous HCT116 cells were arrested in the G_1 phase in response to β-ionone treatment in a dose-dependent manner and there was a corresponding reduction in the S phase. Thus, β-ionone treatment blocks the progression from G_1 to S phase. There was a decrease in the percentage of S-phase cells from 40% to 16% and an increase in the percent of cells with 2N DNA content from 41% (control) to >73% (60 μmol/L β-ionone). Further, a consistent G_1 transition phase arrest was observed in the HCT116 colon cancer cells.

β-Ionone Enhances RXR-α mRNA Expression

RXR-α receptors play an important role in cell growth and induction of apoptosis (23). As illustrated in Fig. 3, treatment of HCT116 cells with β-ionone (for 24 h) stimulated mainly RXR-α receptors mRNA expression, and this induction is dose-dependent. Treatment with 60 μmol/L β-ionone resulted >2.5 fold up-regulation of RXR-α mRNA expression levels in HCT116 cells. Also, our results suggest that β-ionone had no effect on RXR-β mRNA up-regulation (data not shown).

Maximum Tolerated Dose and General Observations of *In vivo* Experiments

Rats were exposed to five different dietary doses, ranging from 0.04% to 0.2% β-ionone for 6 weeks to determine any negative effect on body weight gain, eating habits, and/or overtoxicity. Our results suggest that β-ionone at maximum dose (0.2% or 2,000 ppm in diet) does not produce any retardation in body weight gain and observable toxicities. Based on these dose tolerability results, we have tested the 0.1% and 0.2% β-ionone in diet to assess the chemopreventive effects of this agent against the colonic ACF formation. In the ACF study, the initial body weight before interventions with control or β-ionone diets was 98 ± 1.2 g (mean ± SE). At the time of termination, there was no significant difference in body weight of control and treated rats (data not shown). The food intake of animals in the experimental groups did not show any variation.

Inhibitory Effect of β-Ionone against the Azoxymethane-Induced Colon ACF

In rats fed the control diet, azoxymethane-induced 98.5 ± 8.1 (mean ± SE) colonic ACF containing 20% of one crypt foci, 41% of two crypt foci, 18% of three crypt foci, and 21% of four or more crypt foci (Fig. 4*A*). Rats fed β-ionone diet showed significantly lower number of total mean ACF/colon (34-38%; $P < 0.0009$) when compared with rats that were fed control diet (Fig. 4*B* and *C*). Importantly, aberrant crypts containing multicrypt foci (four or more) were reduced significantly (50-55%; $P < 0.0001$) in rats fed the

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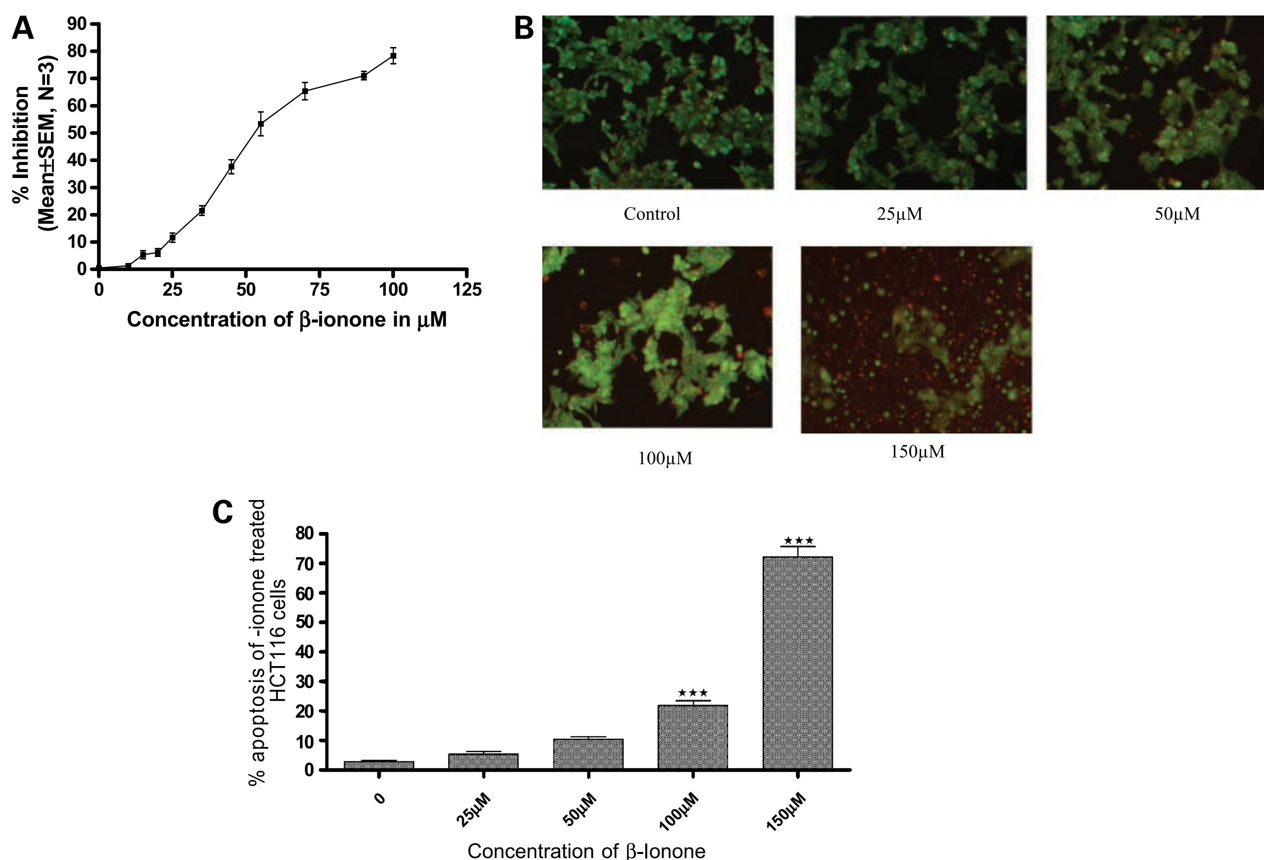

Figure 2. **A,** effect of β-ionone after 24 h of treatment on HCT116 cell proliferation as measured by *N*-acetyl-β-D-hexosaminidase assay. The color formed due to enzyme activity on addition of enzyme substrate is read at 405 nm in microplate reader. IC_{50} is calculated based on 50% inhibition of cell proliferation at a specific concentration of β-ionone. *Points,* mean of three independent experiments; *bars,* SEM. **B,** detection of apoptosis in β-ionone-treated HCT116 cells by ethidium bromide/acridine orange staining. Live cells stain uniformly green and can be distinguished from apoptotic cells, which exhibit yellowish dots of condensed chromatin. Apoptotic cells that have lost their membrane integrity appear orange/reddish orange due to costaining with ethidium bromide. **C,** effect of β-ionone on HCT116 cells apoptosis when incubated with β-ionone for 24 h. *Points,* mean of three independent experiments; *bars,* SE. ***, $P < 0.0002$ and $P < 0.0009$, significantly different from control by unpaired *t* test with Welch's correction (100 and 150 μmol/L, respectively).

β-ionone diet. Although, both 0.1% and 0.2% β-ionone diets induced significant inhibition of azoxymethane-induced colonic ACF; however, our results fail to show any dose-response effect.

RXR-α Expression in Colon Tumors

To understand the relevance of RXR-α role in colon carcinogenesis, we assessed the levels of RXRs in azoxymethane-induced rat colonic tumor tissues and normal appearing colonic mucosa by real-time PCR and immunohistochemistry methods (Fig. 5*A* and *B*). As shown in the Fig. 5*A*, azoxymethane-induced colonic adenocarcinomas showed ~25-fold down-regulation of RXR-α mRNA levels when compared with tumor adjacent normally appearing colonic mucosa. Further, the down-regulation of RXR-α

Table 1. Cell cycle distribution of HCT116 cells by flow cytometric analysis

Treatment	G_0-G_1	*P**	S	*P*	G_2-M	*P*
Control	41.18 ± 0.87†		40.10 ± 0.66		18.17 ± 0.44	
20 μmol/L	59.10 ± 0.66	0.0005	32.00 ± 0.57	0.0027	7.93 ± 0.17	0.0021
40 μmol/L	63.76 ± 0.63	0.0002	29.86 ± 0.46	0.0011	8.03 ± 0.03	0.0019
60 μmol/L	73.93 ± 1.09	0.0002	16.20 ± 0.76	0.0002	10.17 ± 0.44	0.0002

NOTE: DNA content of HCT116 cells after 24-h treatment with β-ionone was determined by propidium iodide labeling.
*Significantly different from control by unpaired *t* test with Welch's correction.
†Percentages of mean ± SE of triplicate analysis from cytometric determinations.

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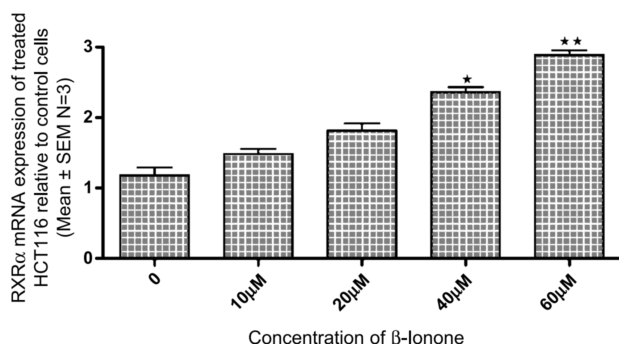

Figure 3. Real-time PCR analysis for RXR-α mRNA expression in β-ionone-treated HCT116 cells. A dose-dependent increase in RXR-α mRNA expression is observed on β-ionone treatment. *Points,* mean of three independent experiments; *bars,* SE. *, $P < 0.011$; **, $P < 0.0007$, significantly different from control by unpaired *t* test with Welch's correction (40 and 60 μmol/L, respectively).

receptors expression in colonic adenocarcinomas has been confirmed by immunohistochemistry. As shown in Fig. 5*B*, normal colonic crypt epithelial cells stained highly positive for RXR-α expressions, whereas colonic epithelial cells in tumors stained very poorly.

Discussion

Dietary factors play an important role in human health and in the development of certain chronic diseases, including cancer (24, 25). Whereas genetic and environmental factors have been recognized as the causatives in the etiology of colonic carcinoma, dietary factors play a major role (26). Epidemiologic studies suggest that presence of minor constituents or non-nutrients are responsible for antimutagenic and anticarcinogenic properties of diets (27). Among the various classes of phytochemicals, carotenoids have been shown to have beneficial effects against many disorders. The major objective of this study was to test the chemopreventive and anticancer effects of naturally occurring phytochemical, β-ionone. We explored the colon cancer chemopreventive properties of β-ionone, a precursor molecule for the synthesis of carotenoids and somewhat similar in structure to retinol (Fig. 1). The rate of tumor growth is dependent on a balance between the rates of cell proliferation and apoptosis. Our results suggest that β-ionone suppresses cell proliferation and induces apoptosis in a dose-dependent manner in human colon cancer cell lines (Fig. 2*A* and *B*). It has been well established that excessive proliferation and lack of apoptosis leads to colon tumor growth, and our results clearly suggest that β-ionone can be used as a potential antitumorigenic agent. A recent study provided a list of 179 volatile isoprenoid constituents of members of seven plant families prominent in western diets. Of these, 41 were screened for tumor suppressive activity *in vitro*; IC_{50}s ranged from 28 to over 1,000 μmol/L. In the present study, β-ionone showed IC_{50} of 60 μmol/L (Fig. 2*A*) in suppressing growth of human colon cancer HCT116 cells. These results further support the previous observations, indicating tumor cell inhibitory effects of β-ionone against various other tumor cells (3, 12–17). Also, cell cycle analysis data suggest that β-ionone induce growth arrest at G_1 phase (Table 1). Our results also suggest that β-ionone induced apoptosis in human HCT116 colon cancer cells; however, we are not aware of any previous reports on the effects of this agent on colon cancer cell apoptosis.

RXRs are nuclear hormone receptors that can bind to 9-*cis*-retinoic acid, a vitamin A metabolite, β-carotene, and docosahexaenoic acid, an ω-3 unsaturated fatty acid, and play a significant role in the expression of normal epithelial and squamous tissue growth (28, 29). β-Ionone has a similar structure to 9-*cis*-retinoic acid, vitamin A, and β-carotene and is known to bind RXRs (Fig. 1). Because β-ionone reduced colonocyte proliferation and enhanced apoptosis in HCT116 cells (Fig. 2*A-C*), we assessed the possibility of β-ionone to serve as a ligand for RXR-α activation in colon cells, as these receptors are known to have a profound effect on colon physiology than other RXRs based on available reports. RXR-β was not altered with β-ionone treatment (data not shown). Our results showed dose-dependent increase in RXR-α mRNA expression on β-ionone exposure to HCT116 cells (Fig. 3). These data indicate that β-ionone might act as a RXR agonist in human colon cancer cells. In *in vitro* studies, 9-*cis*-retinoic acid was also determined to be a ligand for the human nuclear retinoic acid receptor, RXR-α (30, 31). With respect to cancer chemoprevention, there is evidence that retinoic acid receptor and RXR selective ligands cooperatively induce apoptosis (32). The underlying mechanisms by which β-ionone exert a chemopreventive effect in the colon have not been elucidated.

Administration of β-ionone in the diet significantly reduced azoxymethane-induced total colonic ACF formation and multicrypt aberrant crypt growth. To date, there are no studies with *in vivo* (colon) cancer models in evaluating anticarcinogenic potential of β-ionone. Thus, our investigation in well-established models of chemically induced colon carcinogenesis is the first study to validate the chemopreventive effects of β-ionone in animal models. An excessive intake of vitamin A and β-carotene appears to have tumor-promoting effects as per the available reports (28, 33–39), whereas in our present maximum tolerated dose animal experiments no toxic effects in rats were observed. This confirms the safe usage of β-ionone at the doses we evaluated. Unlike our previous observations with carotenoids, β-ionone was observed to be effective in inhibiting ACF even at 0.1% and 0.2% dose levels (40). In the present study, administration of β-ionone provided up to 38% inhibition of azoxymethane-induced total ACF formation and suppression of four or more crypts growth up to ≥55%, which clearly suggests the potential colon tumor inhibitory properties of β-ionone (Fig. 4*B* and *C*). A study that evaluated the chemopreventive activities of the β-ionone *in vivo* on hepatocarcinogenesis showed its effect on decreasing total plasma cholesterol levels and inducing DNA damage. This study can be taken as a valid example that explains suitable plasma levels are present,

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which helps β-ionone in causing DNA damage/apoptosis (41). Previous studies have established that ACF containing four or more aberrant crypts correlate with colon tumor outcome. In this context, our previous studies with naturally occurring agents, such as curcumin (42), caffeic acid ester (43), and diosgenin (44), significantly suppressed azoxymethane-induced colon ACF and colon adenocarcinoma in male F344 rats.

Because an increase in RXR-α was observed in HCT116 colon cancer cells dose-dependently when treated with β-ionone, we further assessed the RXR expression in the colon (tumors versus normal mucosa; Fig. 5), where a decrease in RXR-α was observed in tumors. RXR-α is observed to be down-regulated several-fold (~35) in tumors in comparison with normal rat colonic mucosa (Fig. 5*A* and *B*). Several studies have reported that malignant transformation is associated with alterations in the expression of RXRs in gastric and skin tumors (45–47). Diminished RXR-α protein expression is frequently observed in cancer cells, suggesting its role in the development of human cancer (48, 49). In view of the above, a long-term chemopreventive efficacy study using adenocarcinoma as endpoint marker with β-ionone is needed to further evaluate the exact role of RXR-α.

The mechanism by which β-ionone is inhibiting the development of ACF is not fully known; it is likely due to the different modes of action of this agent. At present, we do not know of any particular cellular target(s), except

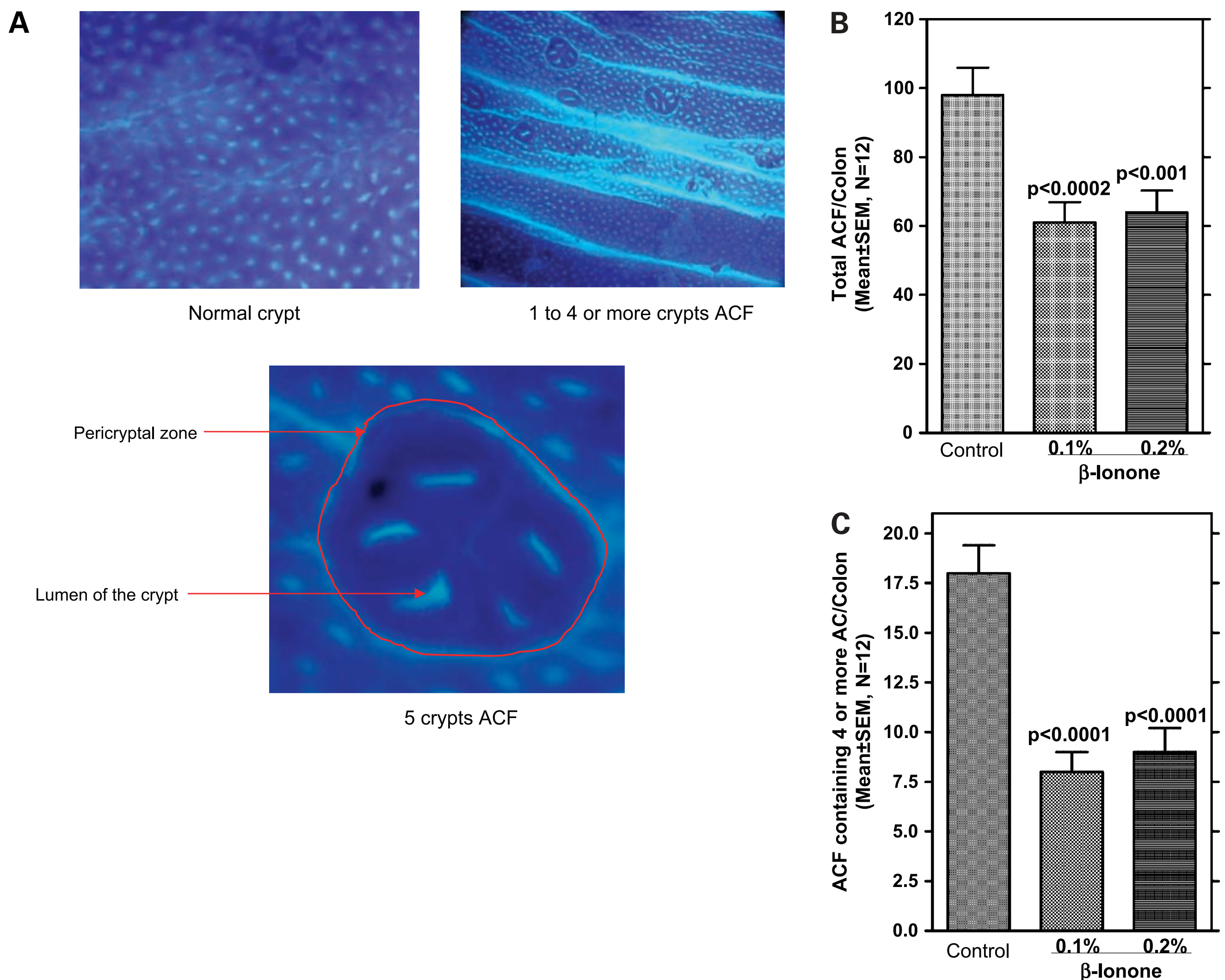

Figure 4. **A,** methylene blue staining of rat colonic tissue showing the normal crypts from rats treated with vehicle and from rats treated with the colon carcinogen azoxymethane. ACFs were distinguished from the surrounding normal crypts by their increased size, increased distance from lamina to basal surfaces of cells, and easily discernible pericryptal zone. **A,** normal crypt (10×), one crypt to four or more crypts (4×), and five crypts ACF (20×). **B,** effect of β-ionone on azoxymethane-induced total colonic ACF formation in male F344 rats. A significant decrease in number of total colonic ACF/colon is observed in rats fed with β-ionone diet. **C,** effect of β-ionone on azoxymethane-induced colonic ACF containing four or more aberrant crypts in male F344 rats. β-Ionone diet fed rats showed a significant decrease in four or more aberrant crypts.

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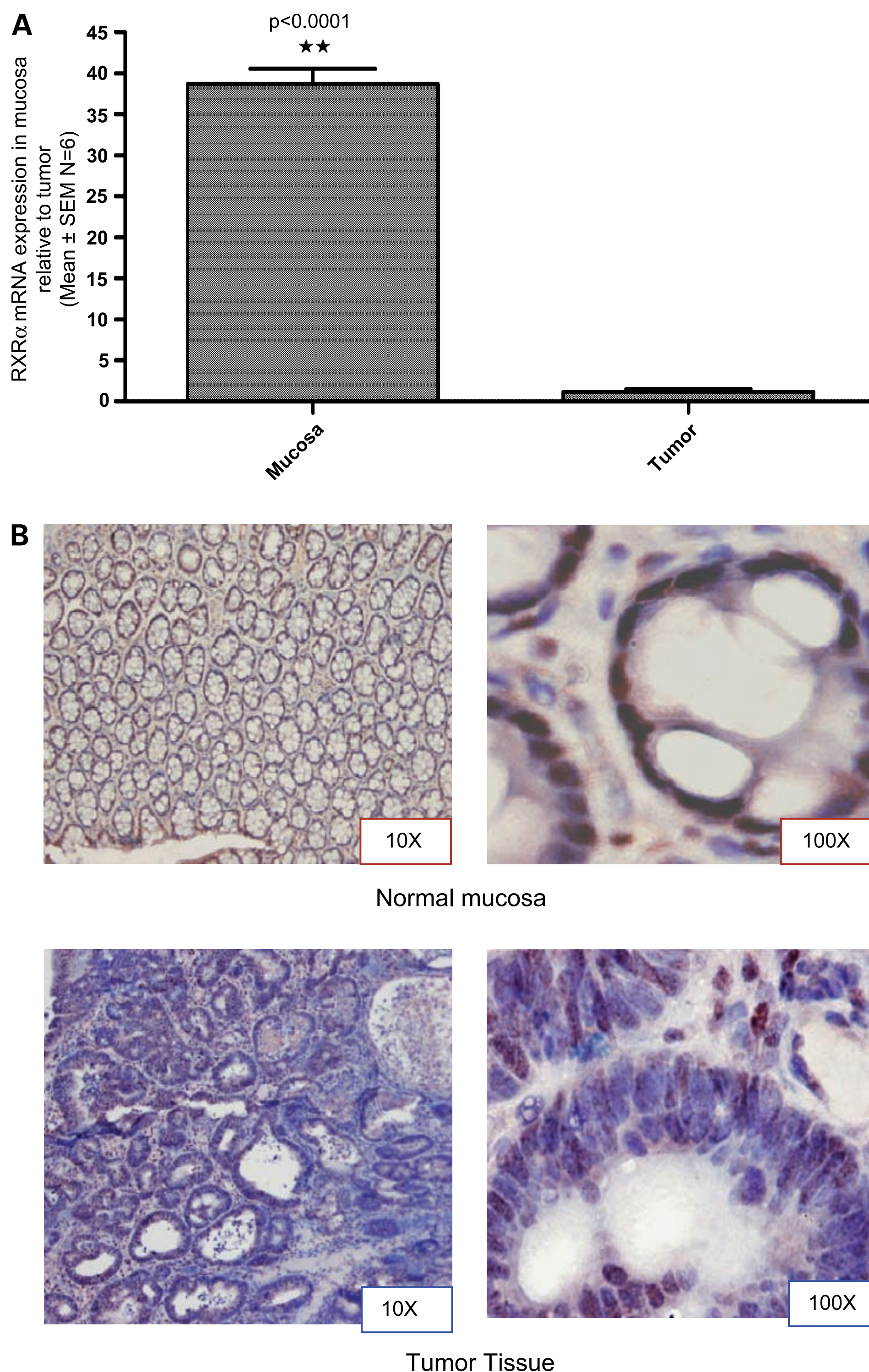

Figure 5. A, down-regulation of RXR-α mRNA expression in azoxymethane-induced colonic adenocarcinomas in comparison with normal mucosa by real-time PCR analysis. *Points,* mean (n = 6); *bars,* SE. Significantly different from tumor in mucosa by unpaired t test with Welch's correction. **B,** immunohistochemistry of RXR-α protein expression in colonic adenocarcinomas and normal colonic mucosa. An intense positive staining is observed in normal colonic mucosa compared with tumor tissue.

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for RXR-α, that would be augmented by this agent. It is well established that regulation of RXR-α may lead to several molecular/cellular changes leading to reduced proliferation and enhanced apoptosis in colon tumor cells. Also, it is possible that β-ionone may provide some antioxidant activities that may help in prevention of carcinogenesis.

Our present data highlight, for the first time, the anticarcinogenic properties of β-ionone *in vivo. In vitro* data indicate that β-ionone suppresses growth and induces

apoptosis in HCT116 cell lines. β-Ionone, being a naturally occurring agent, possesses no toxic effects and is advantageous over other known retinoids, which are known for high toxicity and teratogenicity. These observations suggest that β-ionone would be further developed for colon cancer prevention.

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