β-Escin inhibits colonic aberrant crypt foci formation in rats and regulates the cell cycle growth by inducing p21\textsuperscript{\textit{waf1/cip1}} in colon cancer cells

Jagan M.R. Patlolla, Jayadev Raju, Malisetti V. Swamy, and Chinthalapally V. Rao

Department of Medicine, Hem-Onc Section, OU Cancer Institute, University of Oklahoma Health Sciences Center, Oklahoma City, Oklahoma

Abstract

Extracts of \textit{Aesculus hippocastanum} (horse chestnut) seed have been used in the treatment of chronic venous insufficiency, edema, and hemorrhoids. Most of the beneficial effects of horse chestnut are attributed to its principal component β-escin or aescin. Recent studies suggest that β-escin may possess anti-inflammatory, anti-hyaluronidase, and anti-histamine properties. We have evaluated the chemopreventive efficacy of dietary β-escin on azoxymethane-induced colonic aberrant crypt foci (ACF). In addition, we analyzed the cell growth inhibitory effects and the induction of apoptosis in HT-29 human colon cancer cell line. To evaluate the inhibitory properties of β-escin on colonic ACF, 7-week-old male F344 rats were fed experimental diets containing 0%, 0.025%, or 0.05% β-escin. After 1 week, the rats received s.c. injections of azoxymethane (15 mg/kg body weight, once weekly for 2 weeks) or an equal volume of normal saline (vehicle). Rats were continued on respective experimental diets and sacrificed 8 weeks after the azoxymethane treatment. Colons were evaluated histopathologically for ACF. Administration of dietary 0.025% and 0.05% β-escin significantly suppressed total colonic ACF formation up to ~40% (\textit{P} < 0.001) and ~50% (\textit{P} < 0.0001), respectively, when compared with control diet group. Importantly, rats fed β-escin showed dose-dependent inhibition (~49% to 65%, \textit{P} < 0.0001) of foci containing four or more aberrant crypts. To understand the growth inhibitory effects, HT-29 human colon carcinoma cell lines were treated with various concentrations of β-escin and analyzed by flow cytometry for apoptosis and cell cycle progression. β-Escin treatment in HT-29 cells induced growth arrest at the G\textsubscript{1}-S phase, which was associated with the induction of the cyclin-dependent kinase inhibitor p21\textsuperscript{\textit{waf1/cip1}}, and this correlated with reduced phosphorylation of retinoblastoma protein. Results also indicate that β-escin inhibited growth of colon cancer cells with either wild-type or mutant p53. This novel feature of β-escin, a triterpene saponin, may be a useful candidate agent for colon cancer chemoprevention and treatment.

Introduction

Colon cancer is one of the leading causes of cancer deaths in both men and women in Western countries, including the United States, where about 145,290 new cases of colorectal cancer and 56,290 related deaths are expected for the year 2005 (1). Risk reduction by nutritional intervention holds promise for both prevention and control of colon cancer (2, 3); an alternative approach to identify specific chemopreventive agents holds additional promise (4). Evaluation of food-based, naturally occurring phytochemicals and synthetic agents that can reduce the risk and retard or inhibit the development of colon cancer could lead to new strategies for colon cancer chemoprevention. Phytochemicals or food-based compounds hold promise for cancer chemoprevention and control. Natural products isolated from medicinal herbs have been the potential sources of novel anticaner drugs over the last few decades. The medicinal use of plant extracts seems to be a more natural, less expensive approach and in general involves minimal unwanted side effects. Efficacy studies in the laboratory and preclinical studies have identified several agents with chemopreventive potential in colon cancer that, subsequently, have been used in human clinical trials. Aberrant crypt foci (ACF) are putative preneoplastic lesions of the colons of both animal models and humans (5–7). They serve as intermediate biomarkers to rapidly evaluate the chemopreventive potential of several agents, including naturally occurring agents against colon cancer (5, 8).

Extracts of horse chestnut (\textit{Aesculus hippocastanum}) seed have been used in the treatment of chronic venous insufficiency, hemorrhoids, and postoperative edema (9, 10). β-Escin or aescin (Fig. 1), a triterpene saponin, is one of the major active compounds in the extracts of horse chestnut seed and has been shown to be effective as an alternative to medical treatment for chronic venous...
insufficiency (reviewed in ref. 11). The therapeutic benefit of β-escin is related to the molecular mechanism of the agent, which includes the improved entry of ions into channels (12), thus raising venous tension (13), release of prostaglandin-F₂α from veins (14), antagonism to 5-HT and histamine (15), and its property to decrease the activity of tissue hyaluronidase (16). Some of these properties plausibly make β-escin a prime candidate for a potential cancer chemopreventive agent. For instance, prostaglandin-F₂α strongly inhibited cell proliferation in dimethylhydrazine-induced rat colon adenocarcinoma (17), and compounds with anti-hyaluronidase activity are associated with an increased resistance to tumors (18). Matsuda et al. (19) reported the inhibitory effect of escins isolated from horse chestnut on carrageenan-induced hind paw edema in an animal model for acute inflammation. Moreover, the antiulcerogenic property of escin in a rat model was shown and in part was related to its antisecretory action (20).

Apoptosis, or programmed cell death, is a process essential for normal development and homeostasis in multicellular organisms and is important in controlling cell number and proliferation as part of normal development. Activation of apoptotic pathways is a key mechanism by which cytotoxic drugs kill cancer cells. The precise molecular mechanism of cell cycle regulation by β-escin has not been fully elucidated. Cyclin-dependent kinase (Cdk) complexes are formed and activated at specific stages of the cell cycle, and their activities are required for progression through distinct cell cycle phases. Cell cycle progression is regulated by highly multifaceted proteins that include cyclins and Cdns. Cdk2 and cdk4/6, in association with cyclins E, A, and D, sequentially phosphorylate the retinoblastoma protein and regulate G₁-S phase transition and progression through the S phase (21). These cyclin-Cdk complexes are regulated by the endogenous inhibitor proteins, such as cdk inhibitors p21WAF1/CIP1 and p27KIP1, which inhibit their kinase activities and prevent cell cycle progression (reviewed in refs. 22, 23). In addition, cdk inhibitors are thought to be good target molecules for the development of anticancer agents that can selectively control the cell cycle in cancer cells (24, 25). In the present study, we have assessed the potential chemopreventive efficacy of β-escin on azoxymethane-induced rat colon cancer model using ACF as efficacy marker in male F344 rats. In addition, an attempt was made to understand possible anticancer effects of β-escin in HT-29 colon cancer cell line and to explore the novel feature of this triterpene saponin in selectively blocking the cell cycle events in HT-29 human colon cancer cells.

Materials and Methods

Animals, Care, and Diets

Six-week-old male F344 rats were procured from Charles River Laboratories (Kingston, NY) and housed in suspended cages ~ 10 cm above bedding trays with a 12-hour light/dark cycle in the animal housing facility. Temperature and relative humidity were controlled at 21°C and 55%, respectively. All animals were acclimatized to the above conditions for 1 week with free access to standard laboratory rodent chow and drinking water until initiation of the experiment. Animals were cared for according to the guidelines of the American Council on Animal Care. Diets were based on modified AIN-76A containing 5% corn oil by weight (26). β-Escin (>96% pure) was purchased from Sigma Chemical Co. (St. Louis, MO). The experimental diets contained 0.025% (250 ppm) or 0.05% (500 ppm) of β-escin. Diets were prepared once each week and were stored at 4°C until used. Rats were allowed ad libitum access to the respective diets and tap water.

Experimental Design

Rats at 7 weeks of age were randomized into groups receiving either the control diet or diets containing 0.025% or 0.05% β-escin (n = 18 rats per group; azoxymethane-treated 12 rats plus vehicle-treated six rats). Rats remained on control or experimental diets until termination. At 8 weeks of age, rats intended for carcinogen treatment were s.c. injected with azoxymethane once a week for 2 weeks at a dose of 15 mg/kg body weight. Rats intended for vehicle treatment were given s.c. 0.2 mL of normal saline. All animals were sacrificed by CO₂ asphyxiation 8 weeks after 2nd azoxymethane injection. The colons were removed, flushed with ice cold PBS, and slit open along the length from the anus to the cecum on an ice-cold glass plate. The colons were assessed for any macroscopic changes and were fixed flat between filter papers in 10% buffered formalin for the first 12 hours, then 80% ethanol, and coded for blind scoring.

Quantification of ACF

Topographical analysis of the colonic mucosa according to Bird (27) was done after a minimum of 24 hours in 80% ethanol. Colons were stained with 0.2% methylene blue solution for 5 to 10 minutes, 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 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.

In vitro Studies

Materials. β-Escin, acridine orange, and protease inhibitor were purchased from Sigma (St. Louis, MO); ethidium bromide was purchased from Invitrogen (Carlsbad, CA), and p21, p53, Cdk2, anti-phospho-retinoblastoma protein (Rb; Thr356-R), caspase-3, retinoblastoma (C-15), cyclin D, cyclin E, cyclin A, and actin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

Cell Culture. The human colon cancer cell lines HT-29 and HCT-116 were obtained from the American Type Culture Collection (Manassas, VA) and maintained in McCoy’s 5A medium (HT-29 and HCT-116) with 10% fetal bovine serum with 5% CO2 at 37°C. All experiments were carried out with cells grown to ~70% to 80% confluence. Stock solution of β-escin was prepared by solubilizing 10 mg β-escin in 5 mL of DMSO. Cells were treated with various concentrations of β-escin solution or equal volume of DMSO as vehicle. β-Escin at a concentration of ~30 μmol/L showed significant toxicity in both HT-29 and HCT-116 cell lines. To assess growth inhibition, apoptosis, and molecular markers, we applied various subtoxic dose levels of β-escin, ranging from 0 to 20 μmol/L in human colon cancer cell lines.

Apoptosis Assay by Acridine Orange/Ethidium Bromide Staining. Human colon HT-29 cancer cells cultured for 24 or 48 hours in the presence of various concentrations of β-escin (0, 5, 10, 15, or 20 μmol/L) were washed with PBS and trypsinized. Twenty-five microliters of the cell suspension (~5 × 10^5 per mL) were incubated with 1 μL of 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 AX70 microscope (Tokyo, Japan) connected to a digital imaging system with SPOT RT software version 3.0. Acridine orange is a vital dye that will stain both live and dead cells, whereas ethidium bromide will stain only those cells that have lost their membrane integrity.

Flow Cytometry and Propidium Iodide Staining. Flow cytometry and propidium iodide staining were used to determine the degree of apoptosis and the different phases of the cell cycle as described (28). HT-29 cells (1 × 10^5) treated with different concentrations of β-escin were harvested by trypsinization and fixed in 70% ethanol at 4°C overnight. The cells were subsequently washed once with PBS before the cells were incubated for 5 minutes at room temperature in 1 mL of phosphate-citric acid buffer and then centrifuged (300 × g, 5 minutes). The resulting cell pellet was suspended in 1 mL of DNA-staining solution containing 200 μg of propidium iodide in 10 mL of PBS and 2 mg of DNase-free RNase A. These cells were incubated at room temperature for 30 minutes before being analyzed by flow cytometry for apoptosis and cell cycle. The DNA content was analyzed using FACScan and Cell Quest software (Becton Dickinson, Mountain View, CA).

Western Blot Analysis. Cells exposed to β-escin 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 phenylmethanesulfonyl fluoride, 1 mmol/L DTT, and protease inhibitor cocktail), and the protein content was determined by using the Bio-Rad Protein Assay reagent (Hercules, CA). Separation of proteins (50 μg) was resolved on a SDS-PAGE and transferred onto nitrocellulose membranes. Membranes were blocked with a solution containing 10 mmol/L Tris-HCl (pH 7.5), 150 mmol/L NaCl, 0.01% (v/v) Tween 20, and 5% dry milk and incubated for 1 hour with either anti-p21 (1:250), anti-Cdk2 (1:200), anti-cyclin E (1:1,000), anti-cyclin D (1:1,000), anti-caspase-3 (1:500), anti-Rb (1:1,000), anti-phospho-Rb (1:200), or anti-actin (1:1,000). After washing, the blots with TBST they were then incubated with anti-mouse and rabbit horseradish peroxidase-conjugated secondary antibody followed by washing again with TBST. Washed blots were incubated with Super Signal West Pico Chemiluminescence Substrate (Pierce, Rockford, IL) for 5 minutes and placed on Kodak film.

Statistical Analysis. All data are expressed as mean ± SE. Statistical significance was determined by a two-tailed unpaired t test with Welch’s correction. Differences were considered statistically significant at P < 0.05.

Results

General Observations

To determine if dietary β-escin had any negative effect on body weight gain or eating habits, all rats were monitored on a routine basis. The initial body weight before interventions with control or β-escin diets was 115.4 ± 1.3 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. Our results indicate that 0.025% or 0.05% β-escin were well tolerated and caused no adverse effects in F344 rats for ~10 weeks of chronic feeding.

Effect of β-Escin on Colonic ACF

We used the well-established short-term protocol of the azoxymethane-induced rat colon carcinogenesis model to determine the efficacy of β-escin to inhibit the formation of ACF. Table 1 summarizes the effect of β-escin on azoxymethane-induced aberrant crypt formation. β-Escin at 0.025% and 0.05% given in the diet showed a significant suppression (P < 0.0001) of total colonic ACF by ~40% and 50%, respectively, compared with the control group. As shown in Table 1, β-escin at both dosage levels significantly lowered colonic foci containing one, two, and three crypts.
Importantly, dietary administration of 0.025% and 0.05% \(\beta\)-escin significantly suppressed the formation of large foci (with crypt multiplicity four or more) to 49% (\(P<0.0004\)) and 65% (\(P<0.0001\)), respectively.

\(\beta\)-Escin inhibited the azoxymethane-induced colonic foci containing four or more aberrant crypts in a dose-dependent manner (\(P<0.01\)).

### Induction of Apoptosis

Figures 2 and 3 summarize the effects of \(\beta\)-escin on induction of apoptosis in HT-29 colon cancer cells. Apoptotic cells were visualized in terms of characteristic morphologic changes by the acridine orange/ethidium bromide method. Apoptosis was observed when HT-29 cells were treated with \(\beta\)-escin at 5, 10, 15, and 20 \(\mu\)mol/L concentrations for 48 hours (but not for 24 hours). \(\beta\)-Escin induced apoptosis in HT-29 cells in a dose-dependent manner. As shown in Fig. 2A, live cells are uniformly stained green and can be distinguished from early and late apoptotic cells (Fig. 2B–E). Early apoptotic cells that show condensed chromatin with blebbing was observed in 5 to 10 \(\mu\)mol/L \(\beta\)-escin treatments, and generally, these cells stained as light greenish dots. Late apoptotic cells that have lost their membrane integrity seem orange/red due to co-stain with ethidium bromide. \(\beta\)-Escin at higher concentration (15–20 \(\mu\)mol/L) induced apoptosis in 45% to 69% cells.

To determine whether apoptosis was induced by \(\beta\)-escin in HT-29 cells, we also used a standard flow cytometric assay by measuring sub-G1 DNA content to quantitate the percentage of apoptosis. The percentage of sub-G1 DNA content (fragmented DNA) in the HT-29 cells treated with \(\beta\)-escin was increased in a dose-dependent manner. As shown in Table 2 and Fig. 3A to E, treatment of 20 \(\mu\)mol/L \(\beta\)-escin induced sub-G1 DNA content to 59.15% compared with 8.1% in the control.

### \(\beta\)-Escin Suppresses Proliferation by Arresting the Cell Cycle at G1-S Phase

We examined the effect of \(\beta\)-escin on HT-29 cell cycle by flow cytometry. HT-29 colon cancer cells were exposed to increasing concentrations of \(\beta\)-escin for 24 hours and subsequently processed for cell cycle analysis. Results are summarized in Table 3. We observed that asynchronous HT-29 cells were arrested in the G1-S phase in response to \(\beta\)-escin treatment in a dose-dependent manner, and that

### Table 1. Effect of \(\beta\)-escin on azoxymethane-induced colonic aberrant crypt multiplicity in male F344 rats

| Crypts/focus          | Control diet | 0.025% \(\beta\)-Escin | \(P\)   | 0.05% \(\beta\)-Escin | \(P\)   |
|-----------------------|--------------|-------------------------|--------|------------------------|--------|
| One crypt focus       | 32.4 ± 2.8*  | 17.2 ± 2.1†             | <0.0003| 14.9 ± 1.7†            | <0.0001|
| Two crypt foci        | 62.7 ± 4.6   | 41.3 ± 3.3†             | <0.001 | 36.4 ± 3.3†            | <0.0002|
| Three crypt foci      | 35.0 ± 3.2   | 22.4 ± 1.9†             | <0.003 | 18.3 ± 1.6†            | <0.0003|
| Four or more crypt foci| 29.6 ± 2.9   | 15.1 ± 1.6†             | <0.0005| 10.4 ± 1.1†            | <0.0001|
| Total ACF             | 159.7 ± 11.2 | 96 ± 8.0†               | <0.0002| 80 ± 7.3†              | <0.0001|

*Values are expressed as mean ± SE (\(n=12\) per group).
†Values are significantly different from control group by unpaired \(t\) test with Welch’s correction.

\(P<0.0031\) to 0.0001). Importantly, dietary administration of 0.025% and 0.05% \(\beta\)-escin significantly suppressed the formation of large foci (with crypt multiplicity four or more) to 49% (\(P<0.0004\)) and 65% (\(P<0.0001\)), respectively. \(\beta\)-Escin inhibited the azoxymethane-induced colonic foci containing four or more aberrant crypts in a dose-dependent manner (\(P<0.01\)).

\(\beta\)-Escin at higher concentrations (15–20 \(\mu\)mol/L) induced apoptosis in 45% to 69% cells.

To determine whether apoptosis was induced by \(\beta\)-escin in HT-29 cells, we also used a standard flow cytometric assay by measuring sub-G1 DNA content to quantitate the percentage of apoptosis. The percentage of sub-G1 DNA content (fragmented DNA) in the HT-29 cells treated with \(\beta\)-escin was increased in a dose-dependent manner. As shown in Table 2 and Fig. 3A to E, treatment of 20 \(\mu\)mol/L \(\beta\)-escin induced sub-G1 DNA content to 59.15% compared with 8.1% in the control.

### Figure 2. Acidine orange/ethidium bromide staining of HT-29 cells to detect apoptosis induced by different concentrations of \(\beta\)-escin for 48 h: (A) 0 \(\mu\)mol/L, (B) 5 \(\mu\)mol/L, (C) 10 \(\mu\)mol/L, (D) 15 \(\mu\)mol/L, and (E) 20 \(\mu\)mol/L. Live cells are uniformly green, whereas apoptotic cells are characterized by orange staining due to chromatin condensation and loss of membrane integrity. Magnification, ×400. Triplicate samples were used for each concentration, and in each sample, a minimum of 200 cells were analyzed for apoptosis. Data are presented in Table 2 as % apoptosis (mean ± SE).
there was a corresponding reduction in the S phase. Thus, β-escin treatment blocks the progression from G1 to S phase. There was a decrease in the %S phase from 33% to 10% and an increase in the % of cells with 2N DNA content from 47% (control) to >66% (20 μmol/L β-escin). Furthermore, a consistent G1-S transition phase arrest was observed in the HT-29 colon cancer cells.

We next determined the molecular markers involved in β-escin-induced apoptosis and cell cycle arrest. As shown in Figure 4A, we measured markers, such as caspase-3 and poly(ADP-ribose) polymerase (data not shown) for apoptosis and cyclins A, D1, and E and Cdk-2, p53, p21WAF1/CIP1, and Rb levels for cell cycle regulation. Our analysis of caspase-3 suggests that β-escin does not induce caspase-3 activation (Fig. 4A).

β-Escin Induces p21WAF1/CIP1 and Down-Regulates the Expression of Cyclin A

As we observed whether β-escin arrests the cell cycle in G1-S phase, we investigated whether this arrest results from the selective inhibition of the cyclins that are responsible for cell cycle progression. To understand the mechanism by which β-escin induces G1-S phase arrest, we analyzed the expression levels of several cell cycle regulators (Fig. 4A). β-Escin induced expression of p21WAF1/CIP1 in HT-29 cells, and this induction was dose dependent. In contrast, the expression levels of some of the G1 cell cycle proteins, such as cyclin D1, cyclin E, and Cdk2, remained unaltered. However, cyclin A expression levels were decreased in HT-29 cells treatment with β-escin in a dose-dependent manner.

To investigate whether β-escin-induced p21 expression is dependent on p53 (HT-29 cells have a mutant p53 gene), we tested it in human colon HCT-116 cancer cell line, which carries the wild-type p53 gene. As shown in Fig. 4B, β-escin dose dependently induced the expression of p21WAF1/CIP1 expression without affecting p53 expression levels. These results suggest that wild-type p53 is not essential for β-escin-regulated induction of p21 in colon cell lines. Thus, the p21WAF1/CIP1 induction observed seems to be related to a p53-independent pathway.

β-Escin Decreases the Phosphorylation of Rb

Members of Rb protein are phosphorylated by Cdns (29), leading to activation of gene expression required for cell cycle progression and proliferation. Because p21 is an inhibitor of Cdns, we investigated whether p21 induction by β-escin led to the inhibition of Rb phosphorylation. We studied the phosphorylation status of the endogenous Rb protein particularly for Cdk2 phosphorylation sites (Thr356). As shown in Fig. 4A, Cdk2 phosphorylation sites on Rb were inhibited by β-escin from 10 μmol/L in a dose-dependent manner; however, total Rb protein levels were unaltered.

Discussion

The major objective of this study was to test the chemopreventive and anticancer effects of naturally occurring phytochemicals with implicated medicinal use. Among the various classes of phytochemicals, triterpene saponins have
been shown to have beneficial effects against many inflammatory disorders. In the present study, we explored colon cancer chemopreventive properties of β-escin, a triterpene saponin derived from horse chestnut. To our knowledge, this is the first report that shows β-escin has potential chemopreventive properties against chemically induced colon carcinogenesis. Administration of β-escin in the diet significantly reduced azoxymethane-induced total colonic ACF formation and multicrypt aberrant crypt growth. Previously, several in vitro and in vivo studies supported possible antitumorigenic activity and modulation of inflammatory molecules by β-escin (30). To date, there were no studies with in vivo (colon) cancer models in evaluating anticarcinogenic potential of β-escin. Thus, our investigation in well-established models of chemically induced colon carcinogenesis is the first study to validate our investigation in well-established models of chemically induced colon carcinogenesis. Administration of β-escin in rats will lead to potential antitumorigenic effects. These results further support previous observations indicating tumor cell inhibitory effects of β-escin against the sarcoma, lung, and hepatic cell lines (30). Our results also suggest that β-escin induced apoptosis in human HT-29 colon cancer cells; however, we are not aware of any previous reports on effects of this agent on tumor cell apoptosis.

To understand the mechanism by which β-escin induces antiproliferative and apoptosis, we assessed the capacity of this agent to modify cell cycle progression and apoptosis markers. Flow cytometry results suggest that β-escin induces cell cycle arrests at the G1 phase and at low concentration at the S phase. To further examine this mechanism, we assessed the effect of this agent on the expression levels of G1-S cell cycle relevant molecules. Inactivation of cells by small molecules may occur by two main mechanisms: (a) directly interacting specifically with small molecules with the ATP binding site of Cdk’s or (b) indirectly modulating the upstream pathways that govern

| Treatment   | \( G_{0}/G_{1} \)    | \( P^* \) | \( S \)    | \( P \)   | \( G_{T-M} \)  | \( P \)   |
|-------------|---------------------|---------|----------|---------|--------------|---------|
| Control     | 48.3 ± 0.87*        | <0.0007 | 20.5 ± 0.81* | <0.001 | 12.4 ± 0.63* | <0.01  |
| 5 μmol/L β-escin | 66.6 ± 0.88*   | <0.0006 | 13.4 ± 0.53* | <0.0001 | 15.6 ± 0.75* | <0.06  |
| 10 μmol/L β-escin | 70.0 ± 1.00        | <0.001  | 14.7 ± 0.75* | <0.0002 | 18.6 ± 0.80 | <0.8   |
| 15 μmol/L β-escin | 63.2 ± 0.75*       | <0.0005 | 10.2 ± 0.48* | <0.0001 | 19.3 ± 0.52 | <0.3   |
| 20 μmol/L β-escin | 66.3 ± 0.63*       |         |           |         |              |         |

*Values are significantly different from control by unpaired t test with Welch's correction.

†Values are percentages of mean ± SE of triplicate analysis from flow cytometric determinations.
the Cdk activity (by altering the expression and synthesis of the Cdk/cyclin subunits, Cdns, or endogenous Cdk inhibitors, such as p21WAF1/CIP1; refs. 36–39). As shown (Fig. 4A), we observed that there is an induction of Cdk inhibitor p21WAF1/CIP1 with an associated decrease in the expression of cyclin A, but no modification in cyclin D and cyclin E. Thus, our results at least in part explain G1 arrest by induction of p21WAF1/CIP1 and by inhibiting the cyclin A–dependent kinases (40). These results are consistent with the established hypothesis that overexpression of p21WAF1/CIP1 leads to reduced cell proliferation of mammalian cells (41). Studies have shown that induction of p21WAF1/CIP1 leads to binding Cdk2 at amino acid resides (amino acids 139–164), which is required by cyclin A and E complex (42, 43). In the present study, β-escin induces p21WAF1/CIP1 in a dose-dependent manner with a concomitant decrease in cyclin A levels and no change in the cyclin E levels. Thus, β-escin may directly induce p21WAF1/CIP1, which leads to inhibition of Cdk2 complex formation with cyclins A and E and, or alternatively, reduced levels of cyclin A also limit the Cdk2 complex leading to growth arrest.

Transcription regulation of the p21WAF1/CIP1 gene is regulated by p53-dependent and p53-independent mechanisms (44). In the present study, we observed that β-escin induces p21WAF1/CIP1 expression in a dose-dependent manner in HT-29 cells, which lack functional p53, suggesting an independent mechanism. To further confirm induction of p21WAF1/CIP1 by β-escin, we used human colon HCT-116 cells, which contain wild-type p53 (45). As shown in Fig. 4B, we have observed induction of p21WAF1/CIP1 but not p53 expression levels by β-escin exposure. These results suggest that β-escin-induced p21WAF1/CIP1 expression is independent of p53 status.

The Rb protein family are important substrates of the Cdks and are phosphorylated and dephosphorylated during the cell cycle; the hyperphosphorylated (inactive) form predominates in proliferating cells, whereas the hypophosphorylated (active) form is generally more abundant in quiescent or differentiating cells (46, 47). At least three different cyclin-Cdk complexes have been suggested to phosphorylate Rb during the cell cycle, which includes cdk2-cyclin E in late G1 phase and cyclin A-Cdk2 during the S phase (29, 48, 49). In the present study, β-escin decreases the levels of phospho-Rb in a dose-dependent manner without influencing total Rb protein expression levels. As stated above, β-escin treatment leads to induction of p21WAF1/CIP1 and inhibition of Cdk2-cyclin A or E complex formation, which, in turn, leads to reduced phosphorylation of Rb and arrest observed in HT-29 cells at the G1-S phase. Our results are similar to the recent finding where phenoxodiol (a isoflavone) promotes the specific loss in cellular cdk2 activity and the loss in the phosphorylation of Rb at the Cdk2 (Rb 356) in HN12-treated cells (50).

In the present study, in addition to G1-S phase arrest, we showed that β-escin induces apoptosis in a dose-dependent manner in human colon HT-29 cancer cells. We assessed whether caspase-3 was involved in β-escin-induced apoptosis. As indicated (Fig. 4A), caspase-3 expression levels were not changed, suggesting a caspase-independent mechanism for β-escin-induced apoptosis. The mechanism by which β-escin induces apoptosis in human colon cancer cells needs to be investigated.

In summary, our in vivo studies suggest that dietary β-escin inhibits chemically induced colon carcinogenesis in rats, and our in vitro data indicate that β-escin exhibits cytotoxicity at ~30 μmol/L or above concentrations in colon cancer cell lines. β-Escin at concentrations as low as 5 μmol/L level has been shown to inhibit HT-29 colon cancer cell proliferation. β-Escin induced cell cycle arrest at G1-S phase in part mediated by induction of p21WAF1/CIP1 and/or associated with reduced levels of Cdk2 and cyclin A and E complex and lowered phosphorylation of Rb. Taken together, both in vivo and in vitro assays suggest that the naturally occurring agent β-escin possesses potential colon cancer chemopreventive properties and further studies are warranted.

Acknowledgments

We thank Dr. Ann Thor’s Laboratory and the OMRF Flow Cytometry core for cell cycle analysis and apoptosis and Wade Williams and Julie DeVore for their help in editing and preparation of this article.

References

1. Ahmadin J, Taylor M, Alicia S, Asma G, Elizabeth W, Micheal JT. Cancer Statistics. CA: Cancer J Clinicians 2005;55:6–28.
2. Giovannucci E. Modifiable risk factors for colon cancer. Gastroenterol Clin North Am 2002;31:925 – 43.
3. Milner JA, McDonald SS, Anderson DE, Greenland P. Molecular targets for nutrients involved with cancer prevention. Nutr. Cancer 2001;41:1–16.
4. Conney AH. Tailoring cancer chemoprevention regimens to the individual. J Cell Biochem 2004;91:277 – 86.
5. Bird RP. Role of aberrant crypt foci in understanding the pathogenesis of colon cancer. Cancer Lett 1995;93:55 – 71.
6. Prettow TP, O’Riordan MA, Prettow TG, Stellato TA. Aberrant crypts in human colonic mucosa: putative preneoplastic lesions. J Cell Biochem Suppl 1992;16G:55 – 62.
7. Cheng L, Lai MD. Aberrant crypt foci as microscopic precursors of colorectal cancer. World J Gastroenterol 2003;9:2564 – 9.
8. Corpet DE, Tache S. Most effective colon cancer chemopreventive agents in rats: a systematic review of aberrant crypt foci and tumor data, ranked by potency. Nutr Cancer 2002;43:1–21.
9. Diehm C, Trampisch HJ, Lange S, Schmidt C. Comparison of leg compression stocking and oral horse-chestnut seed extract therapy in patients with chronic venous insufficiency. Lancet 1996;347:292 – 4.
10. Bielanski TE, Piotrowski ZH. Horse-chestnut seed extract for chronic venous insufficiency. J Fam Pract 1999;48:171 – 2.
11. Sirtori CR. Aescin: pharmacology, Pharmacokinetics and therapeutic profile. Pharmacol Res 2001;44:183 – 93.
12. Muraki K, Imaizumi Y, Watanabe M. Ca-dependent K channels in smooth muscle cells permeabilized by β-escin recorded using the cell-attached patch-clamp technique. Pflugers Arch 1992;420:461 – 9.
13. Pearson PJ, Vanhoutte PM. Vasodilator and vasoconstrictor substances produced by the endothelium. Rev Physiol Biochem Pharmacol 1993;122:1 – 67.
14. Berti F, Omini C, Longiave D. The mode of action of aescin and the release of prostaglandins. Prostaglandins 1977;14:241 – 9.
15. Matsuda H, Li Y, Yoshikawa M. Possible involvement of 5-HT and 5-HT2 receptors in acceleration of gastrointestinal transit by escin lb in mice. Life Sci 2000;66:2233 – 8.
16. Facino RM, Carini M, Stefani R, Aldini G, Saibene L. Anti-elastase and anti-hyaluronidase activities of saponins and sapogenins from *Hedera helix*, *Aesculus hippocastanum*, and *Ruscus aculeatus*: factors contributing to their efficacy in the treatment of venous insufficiency. Arch Pharm (Weinheim) 1990;328:20 – 4.

17. Tutton PJM, Barkla DH. Influence of prostaglandin analogues on epithelial cell proliferation and xenograft growth. Br J Cancer 1980;41:47 – 51.

18. Stone OJ. Cancer resistance, carcinogenesis and ground substance viscosity. Med Hypotheses 1986;20:117 – 24.

19. Matsuda H, Li Y, Murakami T, Ninomiya K, Yamahara J, Yoshikawa M. Effects of escins la, lb, lla, and llib from horse chestnut, the seeds of *Aesculus hippocastanum* L., on acute inflammation in animals. Biol Pharm Bull 1997;20:1092 – 5.

20. Marhuenda E, Alarcon de la Lastra C, Martin MJ. Antisecretory and gastroprotective effects of escins in rats. Gen Pharmacol 1994;25:1213 – 9.

21. Nurse P. A long twentieth century of the cell cycle and beyond. Cell 2000;100:71 – 8.

22. Bruce A, Edgar TL, Terry LD. Endoreplication cell cycles: more for less. Cell 2001;105:297 – 306.

23. Grana X, Reddy EP. Cell cycle control in mammalian cells: role of cyclins, cyclin dependent kinases (CDKs), growth suppressor genes and cyclin-dependent kinase inhibitors (CKIs). Oncogene 1998;11:211 – 9.

24. Hunter T. Braking the cycle. Cell 1993;75:839 – 41.

25. Fischer PM, Lane DP. Inhibitors of cyclin-dependent kinases as anti-cancer therapeutics. Curr Med Chem 2000;7:1213 – 45.

26. American Institute of Nutrition. Report of the American Institute of Nutrition ad hoc committee on standards for nutritional studies. J Nutr 1977;107:1340 – 8.

27. Bird RP. Observation and quantification of aberrant crypts in the murine colon treated with a colon carcinogen: preliminary findings. Cancer Lett 1987;37:147 – 51.

28. Zbigniew D, Elzbieta B. Analysis of Apoptotic cells by flow and laser scanning cytometry. Methods Enzymol 2000;322:18 – 39.

29. Zbigniew D, Elzbieta B. Analysis of Apoptotic cells by flow and laser scanning cytometry. Methods Enzymol 2000;322:18 – 39.

30. Bird RP. Observation and quantification of aberrant crypts in the murine colon treated with a colon carcinogen: preliminary findings. Cancer Lett 1987;37:147 – 51.

31. Yang XW, Zhao J, Cui JR, Guo W. Studies on the biotransformation of escin la by human intestinal bacteria and the anti-tumor activities of desacylescins I. Beijing Da Xue Xue Bao 2004;36:31 – 5.

32. Rao CV, Simi B, Reddy BS. Inhibition of cyclin-dependent kinase 2 activity by p53-independent induction of p21WAF1/CIP1. Mol Cancer Ther 2006;5(6). June 2006

33. Rao CV, Desai D, Simi B, Kulkarni N, Amin S, Reddy BS. Inhibitory effect of caffeic acid esters on azoxymethane induced biochemical changes and aberrant crypt foci formation in rat colon. Cancer Res 1993;53:4182 – 8.

34. Rao CV, Desai D, Rivenson A, Simi B, Amin S, Reddy BS. Chemoprevention of colon carcinogenesis by phenethyl-3-methyl caffeate. Cancer Res 1995;55:2310 – 5.

35. Raju J, Patiloma JM, Swamy MV, Rao CV. Diosgenin a steroid saponin of Trigonella foenum graecum (Fenugreek) inhibits azoxymethane-induced aberrant crypt foci formation in F344 rats and induces apoptosis in HT-29 human colon cancer cells. Cancer Epidemiol Biomarkers Prev 2004;13:1392 – 8.

36. Sherr CJ, Roberts JM. CDK inhibitors: positive and negative regulators of G1-phase progression. Genes Dev 1997;13:1501 – 12.

37. Senderowicz AM, Sauville EA. Preclinical and clinical development of cyclin-dependent kinase modulators. J Natl Cancer Inst 2000;92:376 – 87.

38. Senderowicz AM. Small-molecule cyclin-dependent kinase modulators. Oncogene 2003;22:6609 – 20.

39. Senderowicz AM. Cyclin-dependent kinase modulators: a novel class of cell cycle regulators for cancer therapy. In: Giaccione G, Schilske RL, Sondel PM, editors. Cancer chemotherapy and biological response modifiers. Oxford (UK): Elsevier Science; 2001.

40. el-Deyrly WS, Tokino T, Velculescu VE, et al. WAF1, a potential mediator of p53 tumor suppression. Cell 1993;75:817 – 25.

41. Xiong Y, Hannon GJ, Zhang H, Casso D, Kobayashi R, Beach D. p21 is a universal inhibitor of cyclin kinases. Nature 1993;366:701 – 4.

42. Mytosh M, Lung FD, Long YQ, Roller RP, Sikorski RS, O’Connor PMA, p21(Waf1/Cip1) carboxy-terminal peptide exhibited cyclin-dependent kinase-inhibitory activity and cytotoxicity when introduced into human cells. Cancer Res 1999;59:3480 – 8.

43. Cai K, Dynlacht BD. Activity and nature of p21WAF1/CIP1 complexes during the cell cycle. Proc Natl Acad Sci U S A 1998;95:12254 – 9.

44. Gartel AL, Tyner AL. Transcriptional regulation of the p21(WAF1/ CIP1) gene. Exp Cell Res 1999;246:280 – 9.

45. Violette S, Poulain L, Duasaux E, et al. Resistance of colon cancer cells to long-term 5-fluorouracil exposure is correlated to the relative level of Bcl-2 and Bcl-X(L) in addition to Bax and p53 status. Int J Cancer 2002;98:498 – 504.

46. Obaya AJ, Sedivy JM. Regulation of cyclin-Cdk activity in mammalian cells. Cell Mol Life Sci 2002;59:126 – 42.

47. Chen PL, Scully P, Shew JY, Wang JY, Lee WH. Phosphorylation of the retinoblastoma gene product is modulated during the cell cycle and cellular differentiation. Cell 1989;58:1193 – 8.

48. Sherr CJ. Cancer cell cycles. Science 1996;274:1672 – 7.

49. Johnson DG, Schwarz JK, Cress WD, Nevins JR. Expression of cyclin-dependent kinase inhibitors (CKIs). Oncogene 1995;11:211 – 9.

50. Fischer PM, Lane DP. Inhibitors of cyclin-dependent kinases as anti-cancer therapeutics. Curr Med Chem 2000;7:1213 – 45.