Inhibition of Azoxymethane-Induced Colonic Aberrant Crypt Foci Formation by Silibinin in Male Fisher 344 Rats

Balaiya Velmurugan,[1] Rana P. Singh,[1,3] Alpna Tyagi[1] and Rajesh Agarwal[1,2]

Abstract Chemoprevention is a practical approach to control colorectal cancer, which is one of the major causes of cancer mortality in the United States. Based on our recent silibinin efficacy studies in human colorectal cancer cells, we investigated the effects of its dietary feeding on azoxymethane (AOM)-induced aberrant crypt foci (ACF) formation and associated biomarkers in male Fisher 344 rats. Five-week-old male Fisher 344 rats were fed control or silibinin-supplemented (0.033%, 0.1%, 0.33%, or 1%, w/w) diet. After 2 weeks, AOM was injected once a week for 2 weeks while silibinin treatments were continued. In another protocol, identical silibinin treatments were done but started 2 weeks post-AOM initiation. All rats were sacrificed at 16 weeks of age, and colon samples were evaluated for ACF, followed by proliferation, apoptosis, and inducible nitric oxide synthase and cyclooxygenase-2, by immunohistochemistry and/or immunoblotting. Silibinin significantly ($P < 0.001$) reduced dose-dependently the number and multiplicity of AOM-induced ACF formation. Silibinin feeding in pre- and post-AOM initiation decreased mean number of ACF by 39% to 65% and in post-AOM initiation by 29% to 55%. Silibinin dose-dependently decreased AOM-induced colonic cell proliferation, evidenced by proliferative cell nuclear antigen and cyclin D1 immunohistochemical staining, and induced apoptosis in these colon tissues, evidenced by terminal deoxyribonucleotidyl transferase–mediated dUTP nick end labeling staining and cleaved poly(ADP-ribose) polymerase. Furthermore, silibinin significantly decreased AOM-induced inducible nitric oxide synthase– and cyclooxygenase-2–positive cells in colon tissues. The present findings show possible beneficial activity of silibinin at least in early stage of colon tumorigenesis, suggesting that silibinin might be an effective natural agent for colorectal cancer chemoprevention.

Colorectal cancer is one of the most prevalent causes of cancer deaths in developed countries including the United States (1). Recent statistics suggest that 148,810 new cases of colorectal cancer will be diagnosed in 2008, and 49,960 patients would die due to this malignancy in the United States alone (2). The etiology of colon cancer is multifactorial, including familial, environmental, and dietary agents. Despite several advancements in the understanding of the processes in carcinogenesis, presently available therapies, including surgery, radiation, and chemotherapeutic drugs, are still limited for advanced stage colon cancer (3). Nutritional intervention is another effective and promising complimentary strategy for controlling the incidence of colon cancer (4). Several epidemiologic and experimental studies have indicated that plant products exert a protective influence against this disease, and beneficial effects may be partly attributable to polyphenolic phytochemicals, which have a wide range of pharmacologic properties (5, 6). Moreover, the search for putative chemopreventive compounds with minimal toxicity raises particular interest in phytochemicals.

Silibinin, a naturally occurring flavonoid and the major biologically active constituent in milk thistle extract, is one such agent that has shown potential anticancer effects against different cancers in both *in vitro* and *in vivo* systems (7–10). Nontoxicity, even at high doses and longer treatment times, is one of the most important properties of this compound, which has been tested in several animal models using different modes of administration (9, 11). Despite a number of studies convincingly showing the remarkable chemopreventive potential of silibinin in different cancer models, its efficacy against colorectal cancer initiation and development in animal models remains largely unexamined. Although Kohno et al. (12) reported the *in vivo* inhibition of colon carcinogenesis by silibinin-rich mixture silymarin, and previous report from our laboratory has shown the anticancer activity of silibinin in human colon carcinoma HT-29 cells (13), no *in vivo* study

Authors' Affiliations: [1]Department of Pharmaceutical Sciences, School of Pharmacy, and [2]University of Colorado Cancer Center, University of Colorado Denver, Denver, Colorado; and [3]Cancer Biology Laboratory, School of Life Sciences, Jawaharlal Nehru University, New Delhi, India
Received 03/21/2008; revised 05/01/2008; accepted 05/05/2008.
Grant support: USPHS grant RO1 CA112304 from the National Cancer Institute, NIH.
Requests for reprints: Rajesh Agarwal, School of Pharmacy, University of Colorado Denver, Box C-238, 4200 East 9th Avenue, Denver, CO 80262. Phone: 303-315-1381; Fax: 303-315-6281; E-mail: Rajesh.Agarwal@uchsc.edu.

doi:10.1158/1940-6207.CAPR-08-0059

has reported the efficacy of silibinin in colorectal cancer chemoprevention model.

Aberrant crypt foci (ACF) are early morphologic changes observed in rodents after administration of colon-specific carcinogen such as azoxymethane (AOM; ref. 14). Similar lesions were also observed at a high frequency in the colons of the patients with sporadic and inherited forms of colon cancer (15). ACF are considered as putative preneoplastic lesions and are currently used as a surrogate biomarker to rapidly evaluate the chemopreventive potential of several agents, including both naturally occurring and synthetic, using AOM in the Fisher 344 rat model (16–18), which accurately replicates many of the clinical, genetic, cellular, and morphologic features of human colorectal cancer (19). AOM-induced ACF are characterized by an increase in the size of the crypts, the epithelial lining, and the pericryptal zone and share many morphologic and biochemical characteristics with tumors, including a comparable increase in cell proliferation (20).

In the present study, we investigated the possible inhibitory effect of dietary feeding of silibinin, at four different dose levels and in two different phases, on the development of AOM-induced ACF formation in male Fisher 344 rats; sulindac, which is well known as an effective chemopreventive agent in this model (21), was used as a positive control for comparison with silibinin study outcomes. Colonic tissues at the end of the study were also analyzed for proliferation, apoptosis, and inflammation markers. The results of the present study convincingly showed the chemopreventive efficacy of silibinin against AOM-induced ACF in Fisher 344 rats, which is also associated with an *in vivo* decrease in proliferation and inflammation regulators but an increase in apoptotic cells in the colon.

Materials and Methods

Animals and treatment protocol

Male Fisher 344 rats were purchased from The Jackson Laboratory at the age of 4 wk and maintained in the animal housing facility at the University of Colorado Denver. Silibinin was obtained commercially from Sigma Chemical Co., and its purity was analyzed by high-performance liquid chromatography to be >98% (22). The experimental protocol for the present study is shown in Fig. 1. Animals were maintained at 12 h light/12 h dark cycles with free access to water and food (AIN-76A powder diet from Dyets, Inc.). After 1 wk of acclimatization, animals were randomly divided into 12 groups of 10 animals each and fed AIN-76A control diet (groups 1 and 2) or diet supplemented with 0.033%, 0.1%, 0.33%, or 1% (w/w) silibinin (groups 3-6) till the end of the study. Two weeks later, rats in groups 2 to 11 were given s.c. injection of AOM once a week for 2 wk at a concentration of 15 mg/kg of body weight. Two weeks after the last AOM injection, rats in groups 7 to 10 were fed with diet containing 0.033%, 0.1%, 0.33%, or 1% (w/w) silibinin, and the rats in group 11 were fed with 0.032% sulindac. Because sulindac is well known as an effective chemopreventive agent against AOM-induced ACF formation in Fisher 344 rats (21), it was used as a positive control for comparison with silibinin study outcomes. Rats in group 12 were fed with diet containing 1% silibinin alone throughout the study. At 16 wk of age, the rats were sacrificed, and their colons were evaluated for ACF or other marker studies.

Determination of ACF

ACF analysis was done according to Bird (20), in which the colons were longitudinally opened, rinsed with 0.9% NaCl solution, and fixed flat between two pieces of filter paper in 10% buffered formalin for a minimum of 24 h. The colons were then cut into 2-cm segments, starting at the anus, and then stained with 0.2% methylene blue in Krebs-Ringer solution for 5 to 10 min and placed with the mucosal side up on a microscope slide and counted under a light microscope at ×400 magnification. Aberrant crypts were distinguished from the

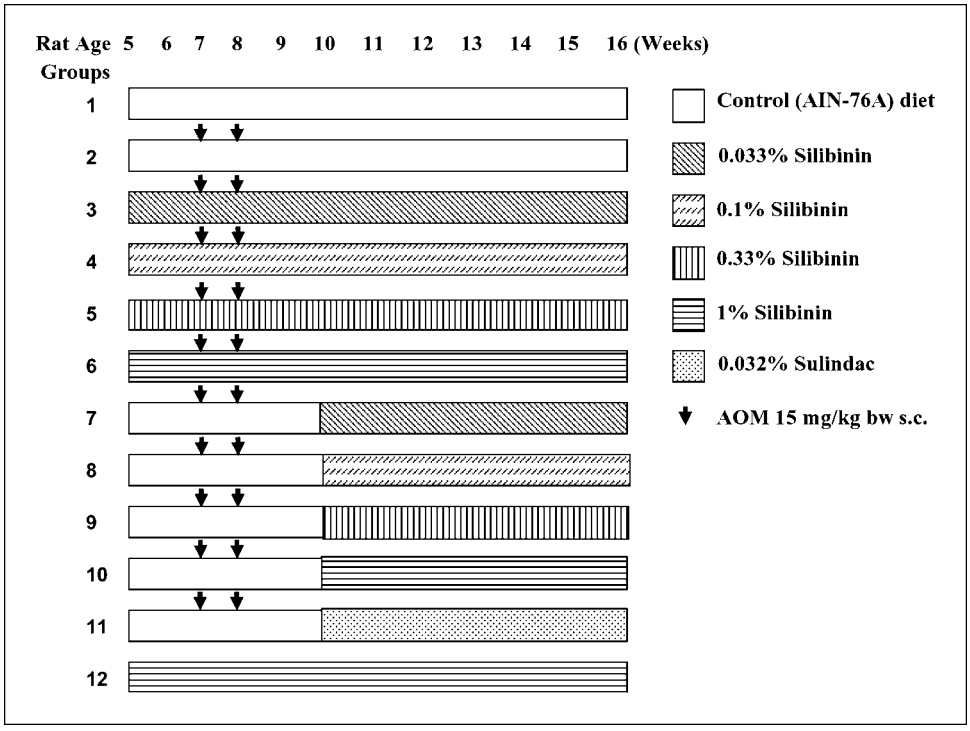

Fig. 1. Experimental protocol for AOM-induced colonic aberrant crypt foci formation in male Fisher 344 rats and chemoprevention studies with silibinin. The animals were randomly divided into 12 groups and fed AIN-76A control diet or diet supplemented with different doses of silibinin. AOM was given once a week for 2 wks at a dose level of 15 mg/kg of body weight by s.c. injection. Other details of the experimental design are described in Materials and Methods.

Table 1. Inhibitory effect of silibinin on AOM-induced aberrant crypt foci incidence and multiplicity in Fisher 344 male rat colon

Groups	Treatment	Incidence of ACF formation (%)	No. ACF/colon	Crypt multiplicity of ACF			
				1 crypt	2 crypts	3 crypts	≥4 crypts
1	Control	0/7 (0)	—	—	—	—	—
2	AOM	7/7 (100)	169 ± 15	71 ± 11	60 ± 7	24 ± 6	14 ± 7
3	0.033% Sb + AOM	7/7 (100)	103 ± 13*	44 ± 5*	30 ± 5*	19 ± 7	10 ± 4
4	0.1% Sb + AOM	7/7 (100)	79 ± 11*	24 ± 9*	26 ± 7*	17 ± 9†	12 ± 4
5	0.33% Sb + AOM	6/6 (100)	68 ± 12*	28 ± 6*	20 ± 5*	12 ± 5*	8 ± 6†
6	1% Sb + AOM	7/7 (100)	60 ± 13*	25 ± 7*	17 ± 8*	9 ± 6*	9 ± 5†
7	AOM + 0.033% Sb	7/7 (100)	120 ± 11*	44 ± 4*	48 ± 3*	15 ± 3*	13 ± 5
8	AOM + 0.1% Sb	7/7 (100)	105 ± 8*	44 ± 4*	39 ± 5*	12 ± 2*	10 ± 2
9	AOM + 0.33% Sb	7/7 (100)	89 ± 9*	34 ± 3*	30 ± 2*	15 ± 2*	10 ± 2
10	AOM + 1% Sb	7/7 (100)	77 ± 8*	29 ± 2*	24 ± 3*	13 ± 3*	11 ± 2
11	AOM + 0.032% sulindac	6/6 (100)	85 ± 7*	32 ± 3*	30 ± 2*	14 ± 3*	9 ± 2
12	1% Sb	0/7 (0)	—	—	—	—	—

NOTE: Data are shown as mean ± SD of seven samples in each group (except groups 5 and 11, $n = 6$).
*$P < 0.001$, vs group 2 (Bonferroni t test).
†$P < 0.05$.

surrounding normal crypts by their increased size, the significantly increased distance from lamina to basal surface of cells, and the easily discernible pericryptal zone. The variables used to assess the aberrant crypts were their occurrence and multiplicity. Crypt multiplicity was determined as the number of crypts in each focus. Multicrypts were categorized as those containing up to ≥4 aberrant crypts per focus.

Immunostaining for proliferative cell nuclear antigen, cyclin D1, inducible nitric oxide synthase, and cyclooxygenase-2

Colon tissue samples were fixed in 10% phosphate-buffered formalin for 10 h at 4°C, dehydrated in ascending concentrations of ethanol, cleared with xylene, and embedded in PolyFin (Triangle Biomedical Sciences). Paraffin-embedded tissue blocks were cut with a rotary microtome into 4-μm sections and processed for immunohistochemical staining. Briefly, after deparaffinization and rehydration, the sections were treated with 0.01 mol/L sodium citrate buffer (pH 6.0) in a microwave for 5 min at full power for antigen retrieval. The sections were then quenched of endogenous peroxidase activity by immersing in 3% hydrogen peroxide for 5 min at room temperature. The sections were incubated with proliferative cell nuclear antigen (PCNA) mouse monoclonal antibody (1:400 dilution; DAKO), cyclin D1 rabbit polyclonal antibody (1:200 dilution; Santa Cruz Biotechnology), inducible nitric oxide synthase (iNOS) rabbit polyclonal antibody (1:200 dilution; Abcam, Inc.), or cyclooxygenase-2 (COX2) rabbit polyclonal antibody (1:100 dilution; Cell Signaling Technologies) in PBS for 2 h at room temperature in a humidity chamber followed by overnight incubation at 4°C. In all the immunohistochemical staining, to rule out the nonspecific staining and allow better interpretation of specific staining at the antigenic site, negative staining controls were used in which sections were incubated with N-Universal Negative Control mouse or rabbit antibody (DAKO) under identical conditions. The sections were then incubated with appropriate biotinylated secondary antibody for 1 h at room temperature followed by 30-min incubation with horseradish peroxidase–conjugated streptavidin. Proteins were visualized with 3,3′-diaminobenzidine for 10 min at room temperature. The sections were counterstained with Harris hematoxylin, dehydrated, and mounted.

Terminal deoxyribonucleotidyl transferase–mediated dUTP nick end labeling staining for apoptotic cells

Apoptotic cells were detected using the DeadEnd Colorimetric terminal deoxyribonucleotidyl transferase–mediated dUTP nick end labeling (TUNEL) system (Promega) following the manufacturer's protocol with some modifications. In brief, the tissue sections after deparaffinization and rehydration were permeabilized with proteinase K (30 mg/mL) for 1 h at 37°C. Thereafter, the sections were quenched of endogenous peroxidase activity using 3% hydrogen peroxide for 10 min. After thorough washing with 1× PBS, the sections were incubated with equilibration buffer for 10 min, and then terminal deoxyribonucleotidyl transferase reaction mixture was added to the sections, except for the negative control, and incubated at 37°C for 1 h. The reaction was stopped by immersing the sections in 2× saline-sodium citrate buffer for 15 min. The sections were then added with streptavidin-horseradish peroxidase (1:500) for 30 min at room temperature, and after repeated washings the sections were incubated with substrate 3,3′-diaminobenzidine until color development (~5-10 min). The sections were then mounted after dehydration and observed under ×400 for TUNEL-positive cells (brown color).

Preparation of tissue homogenates and Western blotting

The colonic tissues were scrapped, and the samples thus obtained were homogenized in lysis buffer using a polytron homogenizer and then centrifuged at 14,000 rpm (8). The supernatants thus obtained were used in the analyses. For each sample, 50 to 80 μg of protein per sample were resolved on Tris-glycine gel, transferred onto nitrocellulose membranes, and blocked for 1 h at room temperature with 5% nonfat dry milk. The membranes were then incubated with the primary antibody anti–cleaved poly(ADP-ribose) polymerase (PARP; Signaling Technologies) overnight at 4°C and then with appropriate secondary antibody. Protein was visualized with the enhanced chemiluminescence detection system. Membranes were stripped and reprobed with anti–β-actin antibody (Sigma) as loading control. The bands were scanned with Adobe Photoshop 6.0 (Adobe Systems), and the mean density of each band was analyzed by the Scion Image program (NIH) and presented as fold change of AOM group below each band.

Immunohistochemical and statistical analyses

All the microscopic analyses were done using Zeiss Axioscop 2 microscope (Carl Zeiss). The pictures were taken with Kodak DC290 camera under ×400 magnification and processed by Kodak Microscopy Documentation System 290 (Eastman Kodak Company). The mean ± SE values were obtained from the evaluation of multiple fields in each group. For each animal, 5 to 10 representative fields were counted at ×400 magnification, and the data represent the results from at least six rats in each group. For statistical significance of the difference, the data were analyzed using the SigmaStat 2.03 software. The statistical significance of difference between control and AOM-treated groups and between AOM-treated and silibinin plus AOM– or AOM plus silibinin–treated groups was determined by one-way ANOVA followed by Bonferroni *t* test for multiple comparisons. $P < 0.05$ was considered statistically significant.

Results

General observations

All rats were monitored on a regular basis to investigate if dietary silibinin had any negative effect on the body weight gain or diet consumption. The food consumption (grams per day per rat) and gain in body weight did not differ significantly betweeen the control and silibinin-fed groups till the end of the study (data not shown). Furthermore, at necropsy, no pathologic alternations were found in any organs by gross observation, including the liver in Fisher 344 male rats.

Suppressive effects of dietary silibinin on ACF formation

A well-established short-term protocol was used to determine the efficacy of silibinin in inhibiting AOM-induced ACF formation. Table 1 summarizes the effect of dietary silibinin on AOM-induced ACF formation. All the rats belonging to groups 2 to 11, which were treated with AOM, developed ACF. The mean number of ACF per colon in the animals given AOM alone (group 2) was 169 ± 15. The dietary administration of silibinin at all four different doses (0.033%, 0.1%, 0.33%, or 1%) given pre- and post-initiation (groups 3-6) or post-initiation alone (groups 7-10) significantly ($P < 0.001$) reduced ACF formation in a dose-dependent manner compared with the group 2 animals. Within two different silibinin treatment protocols, the percentage of inhibition was more in animals given silibinin pre- and post-initiation (39-65% reduction) than

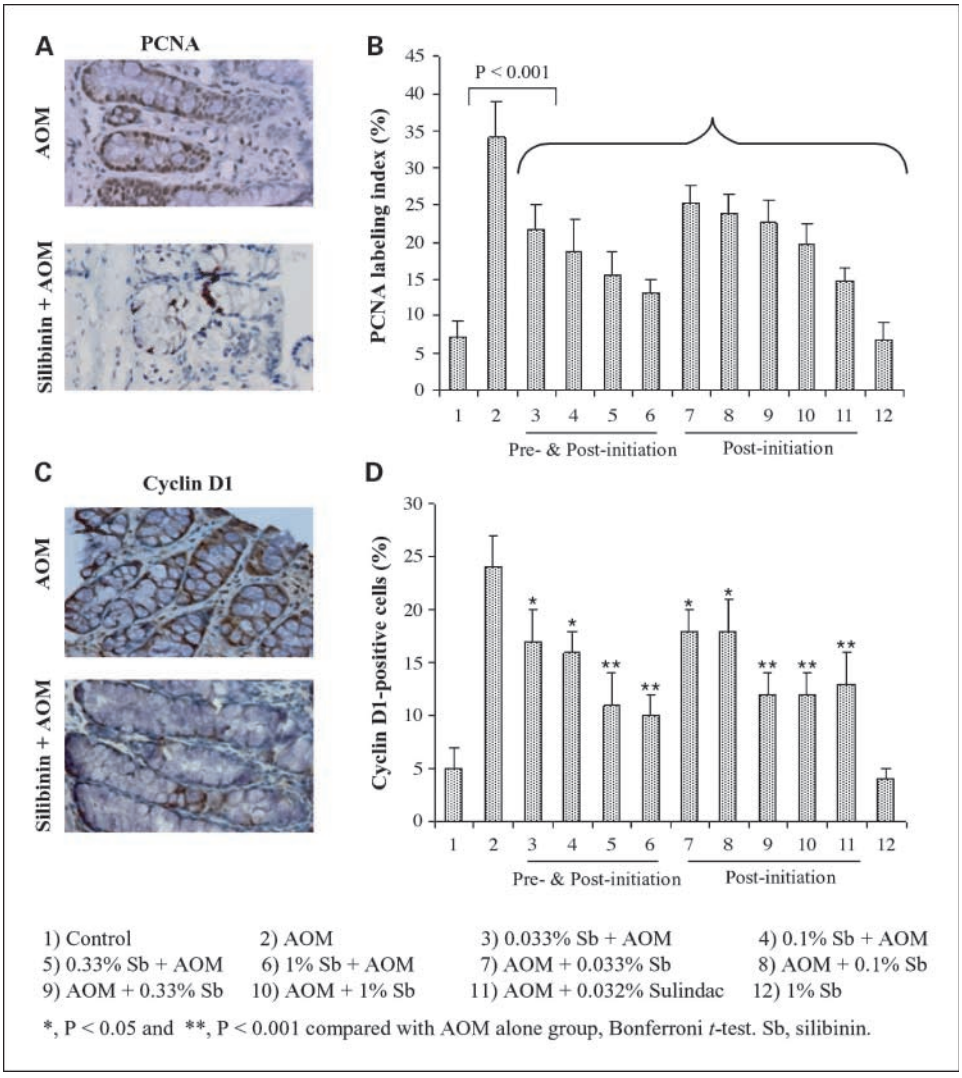

Fig. 2. Inhibitory effects of dietary silibinin on PCNA labeling index and cyclin D1 expression in colon tissues from AOM-exposed rats. Representative photographs of immunohistochemical staining of PCNA-positive (*A*) and cyclin D1–positive (*C*) cells in AOM alone– and silibinin + AOM–treated groups, respectively, are shown at ×400 magnification. Percentages of PCNA-positive (*B*) and cyclin D1–positive (*D*) cells as assessed by quantification of immunohistochemically stained rat colonic epithelium in 5 to 10 randomly selected fields from each tissue sample. *Columns*, mean from at least six rats in each group; *bars*, SE.

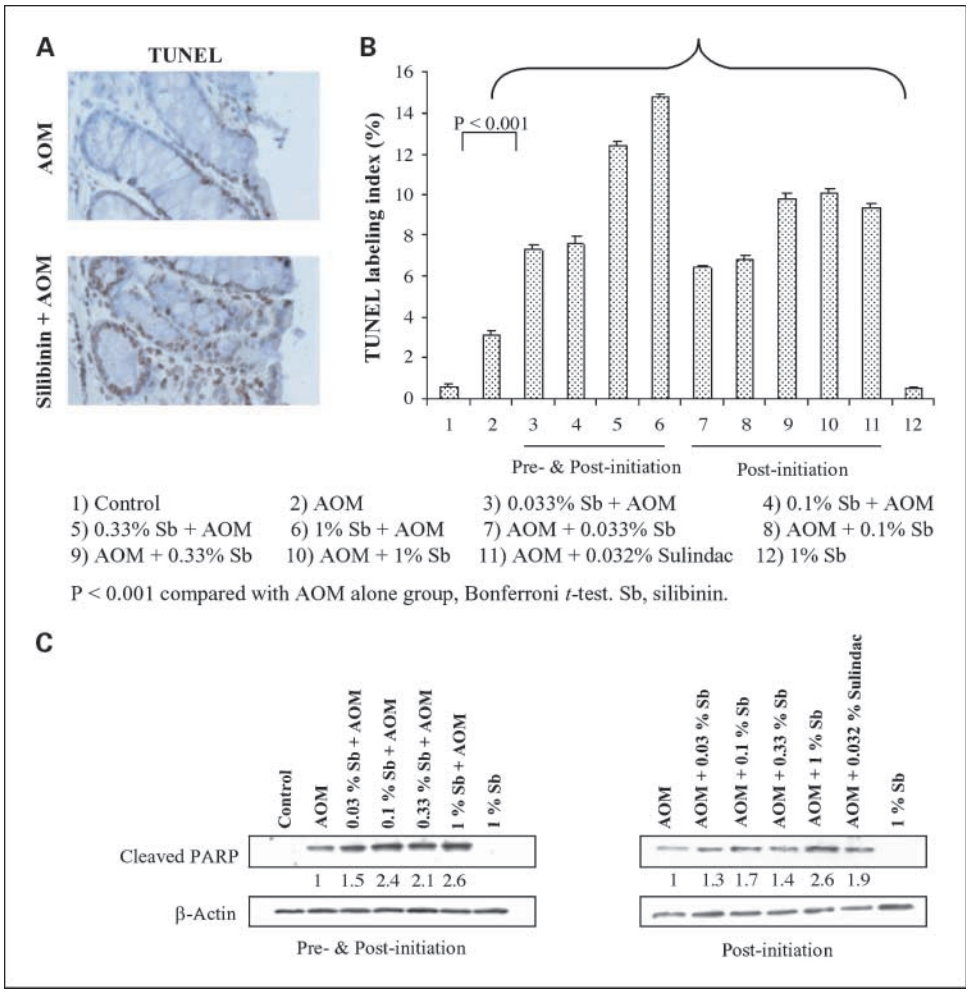

Fig. 3. Effect of dietary silibinin on TUNEL labeling index and expression of cleaved PARP in colon tissues from AOM-treated rats. Colon tissues were analyzed and quantified for apoptosis by TUNEL staining as detailed in Materials and Methods. *A*, representative photographs of the TUNEL-positive cells in AOM alone– and silibinin + AOM–treated groups are shown at ×400 magnification. *B*, percentage of TUNEL-positive cells as assessed by quantification of stained colonic tissues in 5 to 10 randomly selected fields from each tissue sample. *Columns*, mean from at least six rats in each group; *bars*, SE. *C*, the colon tissues collected at the end of the experiment were also analyzed for cleaved PARP expression by SDS-PAGE and immunoblotting as described in Materials and Methods. The densitometric analysis results are shown below each band, representing fold change versus AOM alone.

post-initiation only (29-55% reduction). Furthermore, the number of ACF consisting of >4 crypts also decreased significantly ($P < 0.05$) in silibinin-fed rats (groups 3-10) as compared with AOM alone–treated rats (group 2; Table 1). The animals fed with 0.032% sulindac also showed 50% inhibition in ACF formation compared with group 2 rats (Table 1).

Inhibitory effects of dietary silibinin on PCNA labeling index and cyclin D1–positive cells

The PCNA labeling index and cyclin D1 expression in colonic mucosa are presented in Fig. 2. PCNA is an auxiliary protein of DNA polymerase δ, and high levels of its expression correlate with cell proliferation, suggesting that PCNA is an excellent marker of cellular proliferation (23). Microscopic examination of colonic tissue sections stained for PCNA (brown color) clearly showed a strong staining in AOM-treated samples and its reduction following silibinin treatment, as shown in two representative sections stained immunohistochemically for PCNA (Fig. 2A). Quantitative analyses of all the PCNA-stained sections in different groups (Fig. 2B) clearly showed a significant ($P < 0.001$) increase in the percentage of PCNA-positive cells in the colonic mucosa of AOM-treated rats (34.1 ± 4.9) compared with control (group 1) rats (7.3 ± 2.03); both groups received AIN-76A control diet alone. All the experimental diets containing different dose levels of silibinin (0.033%, 0.1%, 0.33%, or 1%) given either pre- and post-initiation or post-initiation alone, or 0.032% sulindac, significantly ($P < 0.001$) decreased the PCNA labeling index in the colonic mucosa of AOM-treated rats compared with group 2 (Fig. 2B). The PCNA-positive cells were reduced dose-dependently by 37% to 61% and 26% to 42% in different groups fed silibinin in pre- and post-initiation or only post-initiation protocol, respectively (Fig. 2B). In other assays where cyclin D1 levels were analyzed immunohistochemically, compared with the AOM-alone group, the silibinin plus AOM group of samples showed a marked reduction in cyclin D1 staining, as evidenced by representative sections in Fig. 2C. In terms of quantitative analyses of these results, similar to PCNA labeling index, the percentage of cyclin D1–positive cells also significantly ($P < 0.001$) increased in the colonic mucosa of AOM-treated animals (24.3 ± 3) compared with control (group 1) animals (5.1 ± 2; Fig. 2D). A significant decrease ($P < 0.05$ to $P < 0.001$) in cyclin D1 expression was also observed in the animals that received different dose levels (0.033%, 0.1%, 0.33%, or 1%) of silibinin given either pre- and post-initiation or post-initiation alone, compared with AOM-alone rats (Fig. 2D). In quantitative analysis, compared with AOM alone, the percentage of cyclin D1–positive cells

reduced by 29% to 57% and 23% to 50% in different silibinin-fed groups during pre- and post-AOM and post-AOM initiation protocols, respectively (Fig. 2D). The dietary administration of 0.032% sulindac also significantly ($P < 0.001$) reduced the number of cyclin D1–positive cells by 43% compared with AOM-alone rats (Fig. 2D). The percentages of PCNA- and cyclin D1–positive cells were almost comparable between control diet–fed (group 1) and 1% silibinin alone–fed (group 12) rats.

Apoptosis-inducing effects of dietary silibinin

The apoptotic response of dietary silibinin in the colonic tissue of AOM-injected rats was next investigated by TUNEL staining. Representative photographs for TUNEL-positive cells in AOM alone– and silibinin plus AOM–treated groups, shown at ×400 magnification, clearly document that silibinin causes a strong apoptotic effect (Fig. 3A). Quantitative analyses of the stained sections in different treatment groups showed a significant ($P < 0.001$) increase in the number of TUNEL-positive cells by dietary silibinin at all dose levels in AOM-injected rats compared with control diet–fed AOM-injected rats (Fig. 3B). However, the apoptotic induction by silibinin was more profound in the rats given silibinin at pre- and post-initiation (2.4- to 4.8-fold increase) than in the rats given the same dose level of silibinin at post-initiation alone (2.0- to 3.3-fold increase; Fig. 3B). The dietary administration of 0.032% sulindac also caused a 3.0-fold increase in TUNEL-positive cells compared with AOM-alone rats (Fig. 3B). The apoptosis-inducing effect of silibinin was further confirmed by analyzing cleaved PARP expression with Western blotting (Fig. 3C). Similar to the observation in TUNEL staining, dietary silibinin given at pre- and post-initiation or post-initiation phase showed a higher expression of cleaved PARP compared with the AOM-alone group, with a maximum effect at the 1% dose level (2.6-fold). The dietary administration of 0.032% sulindac also increased the expression of cleaved PARP compared with the AOM-alone group.

Inhibitory effects of dietary silibinin on iNOS and COX2 expression

Representative photographs of the immunohistochemical staining of iNOS-positive (Fig. 4A) and COX2-positive (Fig. 4C) cells in AOM alone– and silibinin plus AOM–treated groups clearly show that silibinin decreases the protein levels of these two key molecules in colonic mucosa. Quantitative analyses of the immunohistochemically stained colonic tissues

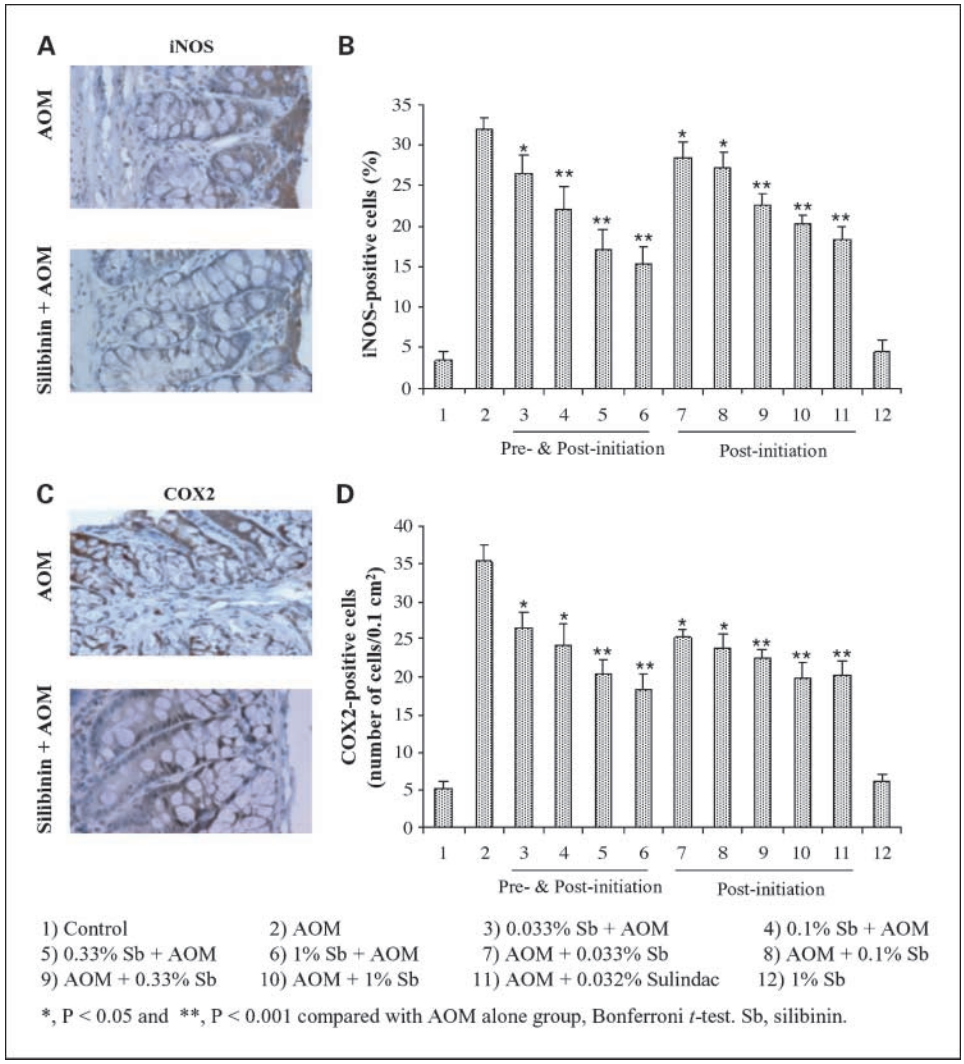

Fig. 4. Inhibitory effects of dietary silibinin on iNOS and COX2 expression in colon tissues from AOM-exposed rats. Representative photographs of immunohistochemical staining of iNOS-positive (*A*) and COX2-positive (*C*) cells in AOM alone– and silibinin + AOM–treated groups, respectively, are shown at ×400 magnification. Percentages of iNOS-positive (*B*) and COX2-positive (*D*) cells as assessed by quantification of immunohistochemically stained rat colonic epithelium in 5 to 10 randomly selected fields from each tissue sample. *Columns,* mean from at least six rats in each group; *bars,* SE.

showed a significantly increased number of iNOS-positive (~9-fold, Fig. 4B) and COX2-positive cells (~7-fold; Fig. 4D) in AOM alone–injected rats compared with those fed with control diet alone. However, iNOS expression was significantly decreased in the animals that received different dose levels (0.033%, 0.1%, 0.33%, or 1%) of silibinin given either pre- and post-initiation or post-initiation alone compared with AOM-alone rats. The reduction in the percentage of iNOS-positive cells was 17% to 52% and 11% to 36% in different silibinin-fed groups during pre- and post-AOM and post-AOM initiation protocols, respectively (Fig. 4B). The administration of 0.032% sulindac in diet also significantly reduced iNOS expression by 43% compared with AOM-alone rats (Fig. 4B). Similar to iNOS, all the dose levels of silibinin given at pre- and post-initiation or post-initiation phase significantly decreased (25% to 48% decrease compared with AOM alone) the percentage of COX2-positive cells in the AOM-injected rats compared with AOM alone–injected rats (Fig. 4D). The dietary administration of 0.032% sulindac also decreased the level of COX2 by 43% (Fig. 4D). Silibinin alone at 1% dose level did not show any effect on COX2 levels in colonic mucosa with comparable staining to the control diet alone group of rats (Fig. 4D). In other studies, no significant decrease in COX1-positive cells was observed in rats fed silibinin at lower doses (0.033% and 0.1%) given at pre- and post-initiation or post-initiation phase; however, administration of silibinin at higher dose levels (0.33% and 1%) also significantly decreased the number of COX1-positive cells in the colon of AOM-injected rats compared with AOM alone–injected positive controls (data not shown).

Discussion

ACF, as first described by Bird (19) in 1987, are putative preneoplastic lesions that appear on the colon surface of the rodents after treatment with colon carcinogens such as AOM. A similar kind of lesions were also later characterized in humans, and since then, the AOM-induced ACF model had been the most valuable experimental model for evaluating both naturally occurring and synthetic agents for their colon cancer chemopreventive efficacy. Furthermore, ACF has been proved to be a reliable biomarker in short-term screening assay for colon tumorigenesis in laboratory rodents (24). Therefore, the present study was designed to evaluate the chemopreventive efficacy of silibinin at four different dose levels (0.033%, 0.1%, 0.33%, and 1%) in two different treatment protocols (pre- and post-initiation and post-initiation) using AOM-induced ACF as a biomarker. Furthermore, in view of the shown chemopreventive efficacy of various non-steroidal anti-inflammatory drugs in the AOM-induced ACF model, the activity of silibinin was also compared with the efficacy of sulindac.

The results of our study showed that dietary administration with silibinin at all four dose levels significantly inhibited AOM-induced ACF formation in both pre- and post-initiation phase and post-initiation phase in Fisher 344 rats. The inhibitory effect of silibinin on colon ACF formation was also associated with the reduction in crypt multiplicity especially suppressing the larger crypts (≥4 crypts), which have a greater tendency to progress to malignancy. These findings suggest that dietary silibinin suppresses both pre- and post-initiation phase and post-initiation phase of chemically induced colon carcinogenesis. Most importantly, the rats fed with the diets containing silibinin showed no adverse effects on food consumption and animal growth rate (data not shown). The inhibitory effect of silibinin on ACF formation is consistent with an earlier report showing that several naturally occurring phytochemicals inhibit AOM-induced ACF formation and crypt multiplicity (25). Kohno et al. (12) have also reported that in both short-term and long-term experiments, dietary feeding of silymarin during the initiation or post-initiation phase of AOM-induced colon carcinogenesis reduces the incidence and multiplicity of colonic adenocarcinoma. Several explanations for the inhibitory effects of silibinin on ACF formation by AOM are discussed below, although the exact mechanism remains to be elucidated in future studies.

Increased cell proliferation has long been shown to play a crucial role in the initiation phase as well as the promotion/progression stage of carcinogenesis. In this regard, PCNA is implicated in DNA replication by forming a sliding platform that could mediate the interaction of numerous proteins with DNA, and hence, PCNA is regarded as a reliable biomarker for cell proliferation (26). In the present study, silibinin at all dose levels and at different phases (pre- and post-initiation as well as post-initiation) of treatment significantly reduced the increase in proliferative index caused by AOM treatment in rats fed with the control diet. Previous report from our laboratory suggests that silibinin is a potent suppressor of PCNA-positive tumor cells (27). In addition to PCNA, silibinin also suppressed the AOM-induced elevation of cyclin D1 levels. In comparison with the normal crypts, the up-regulation in the expression of cyclin D1 in AOM-induced ACF was anticipated to also favor the greater proliferation in ACF. In this regard, cyclin D1, a cell cycle regulator that is overexpressed in a variety of human cancers including colon cancer, has been shown to be repressed by several anticancer phytochemicals (28). Therefore, the observed chemopreventive potential of silibinin against AOM-induced ACF might be partly via its antiproliferative effect.

Apoptosis and associated cellular events have a profound effect on the progression from a benign to a malignant phenotype and can be targeted for the therapy of various malignancies including colon cancer (29). Hence, the apoptosis-inducing effect of silibinin was evaluated using TUNEL-positive index during AOM-induced ACF formation. Furthermore, because caspase-3 activation is one of the most important events in apoptosis, and PARP is a major substrate of activated caspase-3, we also examined the levels of cleaved PARP by Western blotting to further confirm the apoptotic response of silibinin. The results of the present study clearly indicate that dietary silibinin induces apoptosis in a dose-dependent manner in the colon tissue of AOM-injected rats. These results are consistent with the apoptosis-inducing effect of silibinin in human HT-29 colon cancer cells, previously reported by us, where we suggested that the proapoptotic effect of silibinin could be attributed to the inhibition of constitutively active mitogenic and cell survival signaling (13). More recently, we have also shown that silibinin decreases the level of survivin with a concomitant increase in activated caspase-3 as an important *in vivo* mechanism for apoptosis induction in the human bladder tumor xenograft model (30). The apoptosis-inducing effects of silibinin have also been reported in several

other *in vivo* and *in vitro* cancer models (31, 32). With regard to current study, whereas our results clearly show an *in vivo* apoptotic effect of silibinin that could, in part, be responsible for its overall efficacy in inhibiting AOM-induced ACF formation in rat colon, more studies are needed in future to define the underlying molecular events leading to *in vivo* apoptosis induction by silibinin in colorectal cancer models.

The role of inflammatory molecules iNOS and COX2 as enhancers of carcinogenesis in many organs including colon is currently receiving increased attention, and therefore, the suppression of highly elevated iNOS and COX2 expressions has become a target for cancer chemoprevention (33–35). Elevation of iNOS and COX2 contributes to pathologic processes such as inflammation, abnormal cell proliferation, and reduced apoptosis that favor the process of carcinogenesis. Accordingly, the anti-inflammatory activity of silibinin was investigated as one of the mechanisms of its efficacy in inhibiting AOM-induced ACF formation in the rat colon. COX2 is induced by cytokines, mitogens, and tumor promoters and mediates the inflammatory process, catalyzing the conversion of arachidonic acid into prostaglandins. Recent investigations have revealed that COX1 and COX2 are overexpressed in colon tumors (34, 35). In particular, it has been suggested that inhibition of COX2 is negatively related with colon cancer risk. Although we did not investigate the mechanisms of the suppressive effect of silibinin on the elevated levels of iNOS and COX2 in AOM-injected rat colons, one possible mechanism could be the silibinin-caused suppression of the transcriptional activities of signal transducers and activators of transcription and nuclear factor κB, as observed in other studies (36); both of these molecules are well known to regulate iNOS and COX2 expression. Furthermore, tumor necrosis factor α is known to activate activator protein 1, which is important in the induction of COX2 and iNOS transcription. Because silibinin inhibits activator protein-1–dependent transactivation (36), some of the inhibitory effects of silibinin on COX2 and iNOS induction may also be mediated by the inhibition of activator protein 1, which would be consistent with other studies showing that several chemopreventive agents suppress both iNOS and COX2 levels by regulating activator protein-1 and nuclear factor-κB signaling (37, 38).

In conclusion, the findings described here show that dietary administration of silibinin in two different protocols (pre- and post-initiation and post-initiation phases) dose-dependently inhibits the formation and development of AOM-induced colonic ACF in Fisher 344 rats. These promising results suggest the importance of conducting further investigations with silibinin in preclinical colon cancer models, especially long-term *in vivo* efficacy studies, to support the clinical usefulness of silibinin against colon cancer development.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

References

1. Hisamuddin IM, Yang VW. Molecular genetics of colorectal cancer: an overview. Curr Colorectal Cancer Rep 2006;2:53–9.
2. American Cancer Society. Colorectal cancer facts and figures. Atlanta (GA): American Cancer Society; 2008.
3. Boursi B, Arber N. Current and future clinical strategies in colon cancer prevention and the emerging role of chemoprevention. Curr Pharm Des 2007;13:2274–82.
4. Hoensch HP, Kirch W. Potential role of flavonoids in the prevention of intestinal neoplasia: a review of their mode of action and their clinical perspectives. Int J Gastrointest Cancer 2005;35:187–95.
5. Kapiszewska M. A vegetable to meat consumption ratio as a relevant factor determining cancer preventive diet. The Mediterranean versus other European countries. Forum Nutr 2006;59:130–53.
6. Higdon JV, Delage B, Williams DE, Dashwood RH. Cruciferous vegetables and human cancer risk: epidemiologic evidence and mechanistic basis. Pharmacol Res 2007;55:224–36.
7. Agarwal C, Tyagi A, Kaur M, Agarwal R. Silibinin inhibits constitutive activation of Stat3, and causes caspase activation and apoptotic death of human prostate carcinoma DU145 cells. Carcinogenesis 2007;28:1463–70.
8. Gu M, Singh RP, Dhanalakshmi S, Agarwal C, Agarwal R. Silibinin inhibits inflammatory and angiogenic attributes in photocarcinogenesis in SKH-1 hairless mice. Cancer Res 2007;67:3483–91.
9. Singh RP, Deep G, Chittezhath M, et al. Effect of silibinin on the growth and progression of primary lung tumors in mice. J Natl Cancer Inst 2006;98:846–55.
10. Gu M, Singh RP, Dhanalakshmi S, Mohan S, Agarwal R. Differential effect of silibinin on E2F transcription factors and associated biological events in chronically UVB-exposed skin versus tumors in SKH-1 hairless mice. Mol Cancer Ther 2006;5:2121–9.
11. Singh RP, Agarwal R. Prostate cancer prevention by silibinin. Curr Cancer Drug Targets 2004;4:1–11.
12. Kohno H, Tanaka T, Kawabata K, et al. Silymarin, a naturally occurring polyphenolic antioxidant flavonoid, inhibits azoxymethane-induced colon carcinogenesis in male F344 rats. Int J Cancer 2002;101:461–8.
13. Agarwal C, Singh RP, Dhanalakshmi S, et al. Silibinin up-regulates the expression of cyclin-dependent kinase inhibitors and causes cell cycle arrest and apoptosis in human colon carcinoma HT-29 cells. Oncogene 2003;22:8271–82.
14. Suzuki R, Kohno H, Sugie S, Tanaka T. Sequential observations on the occurrence of preneoplastic and neoplastic lesions in mouse colon treated with azoxymethane and dextran sodium sulfate. Cancer Sci 2004;95:721–7.
15. Stevens RG, Swede H, Heinen CD, et al. Aberrant crypt foci in patients with a positive family history of sporadic colorectal cancer. Cancer Lett 2007;248:262–8.
16. Asano N, Kuno T, Hirose Y, et al. Preventive effects of a flavonoid myricitrin on the formation of azoxymethane-induced premalignant lesions in colons of rats. Asian Pac J Cancer Prev 2007;8:73–6.
17. Boateng J, Verghese M, Shackelford L, et al. Selected fruits reduce azoxymethane (AOM)-induced aberrant crypt foci (ACF) in Fisher 344 male rats. Food Chem Toxicol 2007;45:725–32.
18. Raju J, Swamy MV, Cooma I, et al. Low doses of β-carotene and lutein inhibit AOM-induced rat colonic ACF formation but high doses augment ACF incidence. Int J Cancer 2005;113:798–802.
19. Takahashi M, Wakabayashi K. Gene mutations and altered gene expression in azoxymethane-induced colon carcinogenesis in rodents. Cancer Sci 2004;95:475–80.
20. 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.
21. Ohishi T, Kishimoto Y, Miura N, et al. Synergistic effects of (–)-epigallocatechin gallate with sulindac against colon carcinogenesis of rats treated with azoxymethane. Cancer Lett 2002;177:49–56.
22. Zi X, Agarwal R. Silibinin decreases prostate-specific antigen with cell growth inhibition via G_1 arrest, leading to differentiation of prostate carcinoma cells: implications for prostate cancer intervention. Proc Natl Acad Sci U S A 1999;96:7490–5.
23. Shimazaki N, Yazaki T, Kubota T, et al. DNA polymerase λ directly binds to proliferating cell nuclear antigen through its confined C-terminal region. Genes Cells 2005;10:705–15.
24. Asano N, Kuno T, Hirose Y, et al. Preventive effects of a flavonoid myricitrin on the formation of azoxymethane-induced premalignant lesions in colons of rats. Asian Pac J Cancer Prev 2007;8:73–6.
25. Volate SR, Davenport DM, Muga SJ, Wargovich MJ. Modulation of aberrant crypt foci and apoptosis by dietary herbal supplements (quercetin, curcumin, silymarin, ginseng and rutin). Carcinogenesis 2005;26:1450–6.
26. Maga G, Hubscher U. Proliferating cell nuclear antigen (PCNA): a dancer with many partners. J Cell Sci 2003;116:3051–60.
27. Singh RP, Sharma G, Dhanalakshmi S, Agarwal C, Agarwal R. Suppression of advanced human prostate tumor growth in athymic mice by silibinin feeding is associated with reduced cell proliferation, increased apoptosis, and inhibition of angiogenesis. Cancer Epidemiol Biomarkers Prev 2003;12:933–9.
28. Lim do Y, Jeong Y, Tyner AL, Park JH. Induction of cell cycle arrest and apoptosis in HT-29 human colon cancer cells by the dietary compound

luteolin. Am J Physiol Gastrointest Liver Physiol 2007;292:66–75.
29. Schmelz EM, Xu H, Sengupta R, et al. Regression of early and intermediate stages of colon cancer by targeting multiple members of the EGFR family with EGFR-related protein. Cancer Res 2007;67:5389–96.
30. Singh RP, Tyagi A, Sharma G, Mohan S, Agarwal R. Oral silibinin inhibits *in vivo* human bladder tumor xenograft growth involving down-regulation of survivin. Clin Cancer Res 2008;14:300–8.
31. Agarwal C, Tyagi A, Kaur M, Agarwal R. Silibinin inhibits constitutive activation of Stat3, and causes caspase activation and apoptotic death of human prostate carcinoma DU145 cells. Carcinogenesis 2007;28:1463–70.
32. Singh RP, Tyagi AK, Zhao J, Agarwal R. Silymarin inhibits growth and causes regression of established skin tumors in SENCAR mice via modulation of mitogen-activated protein kinases and induction of apoptosis. Carcinogenesis 2002;23:499–510.
33. Watanabe K, Kawamori T, Nakatsugi S, Wakabayashi K. COX-2 and iNOS, good targets for chemoprevention of colon cancer. Biofactors 2000;12:129–33.
34. Niho N, Kitamura T, Takahashi M, et al. Suppression of azoxymethane-induced colon cancer development in rats by a cyclooxygenase-1 selective inhibitor, mofezolac. Cancer Sci 2006;97:1011–4.
35. Chan AT, Ogino S, Fuchs CS. Aspirin and the risk of colorectal cancer in relation to the expression of COX-2. N Engl J Med 2007;356:2131–42.
36. Singh RP, Dhanalakshmi S, Mohan S, Agarwal C, Agarwal R. Silibinin inhibits UVB- and epidermal growth factor-induced mitogenic and cell survival signaling involving activator protein-1 and nuclear factor-κB in mouse epidermal JB6 cells. Mol Cancer Ther 2006;5:1145–53.
37. Surh YJ, Chun KS, Cha HH, et al. Molecular mechanisms underlying chemopreventive activities of anti-inflammatory phytochemicals: down-regulation of COX-2 and iNOS through suppression of NF-κB activation. Mutat Res 2001;480-481:243–68.
38. Liang YC, Huang YT, Tsai SH, Lin-Shiau SY, Chen CF, Lin JK. Suppression of inducible cyclooxygenase and inducible nitric oxide synthase by apigenin and related flavonoids in mouse macrophages. Carcinogenesis 1999;20:1945–52.

Cancer Prevention Research

Inhibition of Azoxymethane-Induced Colonic Aberrant Crypt Foci Formation by Silibinin in Male Fisher 344 Rats

Balaiya Velmurugan, Rana P. Singh, Alpna Tyagi, et al.

Cancer Prev Res 2008;1:376-384.