Research Article

Chemopreventive Efficacy of Raloxifene, Bexarotene, and Their Combination on the Progression of Chemically Induced Colon Adenomas to Adenocarcinomas in Rats

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Abstract

Estrogen receptor (ER)-β signaling is associated positively in colon tumor progression, whereas down-regulation or loss of function of retinoid X receptor (RXR)-α occurs in colon tumors. The chemopreventive efficacies of the estrogen antagonist raloxifene and the selective RXR agonist bexarotene were tested individually and in combination, during promotion and progression stages of colon tumorigenesis. Colon tumors were induced in male F344 rats with azoxymethane and at early adenoma stage, groups of rats (36 or 45 per group) were fed diets containing raloxifene (1.5 or 3 ppm), bexarotene (30 or 100 ppm), or their low-dose combinations for 40 weeks. Raloxifene or bexarotene alone significantly suppressed colon adenocarcinoma formation in terms of multiplicities (mean ± SE): control, 3.59 ± 0.25; 1.5 ppm raloxifene, 2.51 ± 0.29 (P < 0.004); 3 ppm raloxifene, 2.14 ± 0.28 (P < 0.0001); 50 ppm bexarotene, 2.25 ± 0.32 (P < 0.001); 100 ppm bexarotene, 2.1 ± 0.27 (P < 0.0001); and 1.5 ppm raloxifene + 50 ppm bexarotene, 1.57 ± 0.21 (P < 0.0001). The low-dose combination caused significant (56%) inhibition of adenocarcinomas as compared with control diet fed rats. Tumors exposed to raloxifene, bexarotene and/or the combination showed significant suppression of proliferating cell nuclear antigen, cyclin D1, and β-catenin with an increased apoptotic cells (3-fold) and p21 expression (3.8-fold) as compared tumors of rats fed control diet. The combination of low doses of raloxifene and bexarotene significantly suppressed the progression of colonic adenomas to adenocarcinomas and may be useful for colon cancer prevention and/or treatment in high-risk individuals. Cancer Prev Res; 6(12); 1251–61. © 2013 AACR.

Introduction

Colorectal cancer is the third most common cancer diagnosed in both men and women in the United States. The American Cancer Society’s most recent 2013 estimates are 102,480 new cases of colon cancer and 40,340 new cases of rectal cancer. Overall, the lifetime risk of developing colorectal cancers is about 1 in 20. Although 90% of the cases of the disease occur in people older than 50 years, studies show that colorectal cancer is on the increase in younger patients. Changes in lifestyle, hormone replacement therapies, and environmental hormone mimics may be playing a role in this increase in colorectal cancer in young adults.

The biologic effects of estrogen are mediated by its binding to one of the structurally and functionally distinct estrogen receptor (ER) drms (ER-α and -β). ER-β is the predominant ER in the human colonic epithelium (1). There are opposing results with respect to the expression and roles of ERs in colon cancer. Some studies reported that the levels of ER-β are reduced in colorectal cancer compared with normal colonic tissue; and it has been suggested that these decreased levels may be related to loss of differentiation and advanced Dukes staging (2). In contrast, we and others have found that ER-β is associated with positive signaling in animal and human colon carcinomas (3–5). Chlebowski and colleagues (6) observed an increased number of positive lymph nodes and more advanced regional and metastatic disease in women who were on hormone treatment compared versus placebo (76.2% vs. 48.5%; P = 0.004) in a Women’s Health Initiative (WHI) trial of estrogen plus progestin in postmenopausal women. Also, more women in the hormone group had metastatic colorectal cancer compared with those taking placebo. Another study reported no statistical difference in colorectal cancer between a hormone therapy group and a placebo group (7). Two other prospective studies reported that high concentrations of circulating estrogen conferred increased risk for colorectal cancer (8, 9). A recent case-controlled study reported that

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men with both an estrogen receptor 2 gene ESR2 genetic variant (rs4986938 risk genotype) and high estradiol level had a higher risk for developing colorectal cancer (10). A recent report by Simon and colleagues (11) showed that an estrogen plus progestin group had no reduced colorectal cancer mortality; rather, they observed a nonsignificant increase in colorectal cancer deaths compared with placebo (37 vs. 27 deaths; P = 0.320). The selective estrogen receptor modulators (SERM) exhibit specific ER agonistic and antagonistic activity by binding to ER-α and/or ER-β (12). Of the SERMs, raloxifene has antiestrogenic effects on the breast and bone, but it does not have an estrogenic effect on the uterus (12). We have reported inhibition of early precursor lesions in colon by raloxifene (3) and others reported inhibition of carcinogen-induced mammary carcinoma (13) in animal models.

Retinoid X receptors (RXR) and retinoic acid receptors (RAR) are nuclear receptors that mediate the biologic effects of retinoids by their involvement in retinoic acid–mediated gene activation. We and others have identified downregulation or loss of function of RXR-α in preclinical and clinical colon tumors (14). We previously have reported increased expression of RXR-α and inhibition of colon tumors in ApcMin/þ mice treated with the selective RXR agonist bexarotene and increased RXR-α (15), and inhibition of azoxymethane-induced colonic ACF formation in F344 rats treated with β-ionone, present in vegetables and fruits (14). Thus, increased RXR-α expression and decreased ER-β expression might favor colon tumor growth. RXR receptor is reported to repress estrogen-responsive genes activated by ER in human breast cancer cells indicating a cross-talk between ER and RXR receptors functions (16). RXRs form heterodimers with various nuclear receptors and bind to their response elements and have the potential to interact with signaling pathways either negatively or positively. Thus, synergy between ER and RXR is not confined to single pathway mechanism. As reported by Suh and colleagues (17), bexarotene suppress ER-positive breast cancers by synergizing with SERMs through TGF-β pathway in rat models. In a colorectal cancer, multiple genes involving various signaling pathways are deregulated. It is noteworthy that colon tumors overexpress ER-β and frequently associated with loss of expression of RXR-α. Hence, developing ER-β antagonist and RXR-α agonist combination is considered to be a rational approach for colon cancer prevention.

U.S. Food and Drug Administration (FDA) had approved raloxifene for treatment of osteoporosis and invasive breast cancer; whereas bexarotene for cutaneous T-cell lymphoma. Bexarotene also has been used off-label for lung cancer, breast cancer, and Kaposi’s sarcoma. As discussed above, molecular pathogenesis of colon tumor progression suggests a progressive loss of RXR-α and an increase in ER-β expression. We hypothesize that upregulation of RXR-α in association with downregulation ER-β expression may provide better chemopreventive efficacy. Here, we evaluated the dose response and combinational chemopreventive efficacies of raloxifene and bexarotene during promotion and progression stages of colon adenocarcinoma formation in F344 rats.

Materials and Methods

Chemicals

Raloxifene and bexarotene were provided by the Division of Cancer Prevention (DCP) Repository at the National Cancer Institute (Rockville, MD). Primary antibodies (monoclonal/polyclonal) to β-catenin, cyclin E, cyclin D1, cdk2, Cdc25c, p21, and proliferating cell nuclear antigen (PCNA) were from Santa Cruz Biotechnology. Hors eradish peroxidase–conjugated secondary antibodies were from Santa Cruz Biotechnology. A human tissue array for colorectal cancer was obtained from Cybrdi.

Immunohistochemistry of a human colorectal adenocarcinoma tissue array for expression of RXR-α and ER-β

A colorectal carcinoma tissue array consisting of 72 formalin-fixed cores from 69 different patients was used to examine protein expression patterns for RXR-α and ER-β. Tissue array sections (5 μm) were dried at 56°C, deparaffinized in xylene (15 minutes twice), rehydrated, and washed with PBS for 15 minutes at room temperature. Specimens were treated in a water bath in 0.01 mol/L citrate buffer (pH 6.0) for 30 minutes at 100°C, slowly cooled to room temperature, and washed with PBS for 5 minutes at room temperature. After quenching endogenous peroxide with 3% hydrogen peroxide in PBS for 10 minutes at room temperature, the sections were incubated with a blocking solution (supplied with the Zymed kit) for 60 minutes at room temperature. Then, the slides were incubated overnight at 4°C with a 1:300 dilution of anti-RXR-α or a 1:200 dilution of anti-ER-β (Santa Cruz Biotechnology). After several washes with PBS, the slides were incubated with secondary antibody for RXR-α and ER-β for 2 hours. The color reaction was developed using 3,3′-diaminobenzidine (DAB) according to the manufacturer’s instructions given in the kit supplied by Zymed Laboratories. The percentage of immunostained cells was graded as follows: 0 (no staining); 1 (>0%–5%); 2 (>5%–50%); and 3 (>50%).

Animals and experimental diets

Weanling male F344 rats obtained from Harlan Breeding Laboratories were randomly distributed by weight into control and experimental groups. Animals had access to food and water at all times. Food cups were replenished with fresh diet twice weekly. All ingredients for the semipurified diets were purchased from Bioserv and stored at 4°C before diet preparation. Diets were based on the modified American Institute of Nutrition (AIN)-76A diet. Modified AIN76A diet contains corn starch 52%, casein 20%, mineral mix 3.5%, dextrose 13%, vitamin mix 1%, cellulose 5%, m-methionine 0.3%, choline bitartrate 0.2%, corn oil 5%. Bexarotene and/or raloxifene was premixed with a small quantity of diet and then blended into bulk diet using a Hobart mixer. Both control and experimental diets were prepared weekly and stored in a cold room. Agent content in
At termination, animals were killed by CO2 asphyxiation. The size of each tumor were noted. Mucosal scrapings were processed for histopathologic analysis. Tissues were removed, snap frozen, or fixed in 10% buffered formalin for 24 hours and transferred to 80% ethanol for further analysis. The dose of raloxifene was based on our previous 8-week study using ACF as an endpoint, and the dose of bexarotene was based on our previous study with Apcmin mice (3, 17). At termination, animals were killed by CO2 asphyxiation and the colons were removed, rinsed in PBS, opened longitudinally, and flattened on a filter paper. The location and size of each tumor were noted. Mucosal scrapings were collected and stored at –80°C for further analysis. Tumors were removed, snap frozen, or fixed in 10% buffered formalin for 24 hours and transferred to 80% ethanol for histopathologic analysis.

Bioassay: chemopreventive efficacy evaluation
Beginning at 5 weeks of age, all rats were fed the modified AIN-76A diet. At 7 weeks of age, the animals were given subcutaneous injections of azoxymethane at a dose of 15 mg/kg body weight or saline as solvent control once weekly for 2 weeks. Nine weeks after the azoxymethane injections, animals were maintained on AIN-76A diet or AIN-76A diet containing 1.5 ppm or 3 ppm of raloxifene, 50 or 100 ppm of bexarotene, or their low-dose combinations for 40 weeks. The dose of raloxifene was based on our previous 8-week study using ACF as an endpoint, and the dose of bexarotene was based on our previous study with Apcmin mice (3, 17). At termination, animals were killed by CO2 asphyxiation and the colons were removed, rinsed in PBS, opened longitudinally, and flattened on a filter paper. The location and size of each tumor were noted. Mucosal scrapings were collected and stored at –80°C for further analysis. Tumors were removed, snap frozen, or fixed in 10% buffered formalin for 24 hours and transferred to 80% ethanol for histopathologic analysis.

Histopathology and immunohistochemistry
The tumor tissues were fixed in 10% formalin, dehydrated, embedded in paraffin, and cut into 4-μm-thick sections. For histopathology, the sections were hydrated and stained with hematoxylin and eosin according to the standard protocol. The stained sections were analyzed by a pathologist blinded to the treatments. Protein markers were evaluated in control and treated sections by immunohistochemical analysis as reported previously. Sections were incubated with primary antibodies against PCNA (1:1,500), cyclin D1 (1:500), β-catenin (1:500), p21 (1:500), ER-β (1:500), and RAR-α (1:500) and then with appropriate secondary antibodies. Random images were taken with a bright-field microscope (Olympus AX71) connected to a digital imaging system with SPOT RT software version 3.0. Scoring of PCNA-positive cells in the tumors was performed by two investigators blinded to the identity of the samples (light microscopy at 400× magnification). Cells with a brown nucleus were considered positive. The proliferation index was determined by dividing the number of positive cells by the total cells and multiplying by 100.

Apoptosis assay
Sections of 5 μm thickness were cut, mounted on slides, rehydrated, and stained using the terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (TUNEL) method as previously described. TUNEL-positive cells were visualized by chromogenic staining with DAB and slides were counterstained with methyl green. Stained apoptotic epithelial cells (a minimum of 10 microscopic fields per section) were counted manually in a single-blind fashion.

Protein expression by Western blot analysis
Tissues exposed (24 hours) to various concentrations of raloxifene, bexarotene and combinations were harvested by gentle scraping and processed for protein and estimated protein content using the Bio-Rad Protein Assay reagent as mentioned previously. An aliquot (50 μg protein/lane) of the total protein was subjected to SDS-PAGE (10% or 15%), and proteins were transferred to nitrocellulose membranes. After blocking in 5% milk, the membranes were incubated with primary antibodies to β-catenin, cyclin E, cdk2, Cdc25c, p21, and PCNA (1:500, in TBS-Tween 20 solution), then probed with horseradish peroxidase–conjugated secondary antibody. Detection was done using the SuperSignalWest Pico Chemiluminescence procedure developed by Pierce. The bands were captured on Ewen Parker Blue sensitive X-ray films. The bands were analyzed by densitometry using imaging software. Immunoblotting with tubulin antibody was done to confirm equal protein loading.

ER-β, β-catenin, cyclin D1, IL-4, and IL-6 messenger RNA expression by reverse transcription PCR
Total RNA from tumor samples was extracted using TRIzol (Ambion) as per the manufacturer’s instructions. Equal quantities of DNA-free RNA were used in reverse transcription reactions to make complementary DNA using SuperScript reverse transcriptase (Invitrogen). PCR was carried out for ER-β, β-catenin, cyclin D1, interleukin (IL)-4, and IL-6 using the following conditions: ER-β primers and conditions were as used in the report by Price and colleagues (18). β-Catenin and IL-4 denaturation was carried out at 94°C for 3 minutes, followed by 35 cycles at 94°C for 30 seconds, 55°C for 1 minute, and 72°C for 1 minute. Oligonucleotide primer sequences used for β-catenin were as follows: 5′-CAGGATCCACAAAGAAAAAGGTCTCTCA-3′ (sense) and 5′-GAGAATTCAGGTCAAGTATCAACCA-3′ (antisense). IL-4 oligonucleotide primer sequences used were as follows: 5′-CAGGATCCACAAAGAAAAAGGTCTCTCA-3′ (sense) and 5′-GAGAATTCAGGTCAAGTATCAACCA-3′ (antisense). IL-4 oligonucleotide primer sequences used were as follows: 5′-GACTGATTGTTGACAGCCACTGC-3′ (antisense) and 5′-TACCGACCACTTCTGTAGCTCTA-3′ (antisense). IL-6 denaturation was done at 94°C for 30 seconds, followed by 35 cycles at 94°C for 30 seconds, 56°C for 1 minute, and 72°C for 1 minute. Cyclin D1 denaturation was done at 94°C for 3 minutes, followed by 35 cycles at 94°C for 30 seconds, 60°C for 30 seconds, and 72°C for 45 seconds. The oligonucleotide primer sequences used for the cyclin D1 gene were as follows: 5′-GACCTGTCCGGTGACGCAT-3′ (sense) and 5′-TACCGACCACTTCTGTAGCTCTA-3′ (antisense). PCR was done using the Taq polymerase Master Mix (Qiagen). The PCR products were visualized and photographed under UV illumination.

Statistical analysis
Tumor multiplicity, defined as mean number of tumors per rat, was analyzed by unpaired Student “t” test with Welch correction. The tumor incidence (percentage of...
rats with colon tumors) was analyzed by Fisher exact two-tailed test. The data are presented as means ± SEM. Differences between body weights were analyzed by ANOVA. Differences between groups are considered significant at \( P < 0.05 \). All statistical analysis was conducted using GraphPad Prism Software 5.1 (GraphPad Software, Inc.).

Results

Expression of RXR-α and ER-β in human colorectal tissue

As a first step toward evaluating the role of RXR-α and ER-β in colorectal cancer, we examined expression of the two receptors in a tissue array consisting of samples from patients (male and female from 25 to 79 years of age) diagnosed with adenocarcinoma, grades 1 to 3. Strong positive staining was seen with an RXR-α antibody in normal mucosal tissue but very weak or no staining was observed in tumors, irrespective of sex or age (Fig. 1A, top). ER-β showed strong/granular staining in tumor tissue with very weak to no staining in normal tissue (Fig. 1A, bottom). The quantitative analysis of ER-β antibody staining showed a majority of patients (94%) colon adenocarcinomas had overexpression (41% score of 3, 35% score of 2, and 17% scored of 1) as compared with patients’ normal appearing colonic mucosa showing without any expression (Table 1). In contrast, quantitative analysis of RXR-α antibody staining showed in all the normally appearing colonic tissue samples; as compared with no expression in 65% of colon adenocarcinomas and a modest (score 1) expression in remaining patient’s colonic tumors (Table 1). These findings clearly suggest that colon tumor progression was associated with overexpression of ER-β and downregulation of RXR-α expression.

Lack of overt toxicity of bexarotene and raloxifene doses

The maximum-tolerated doses (MTD) for raloxifene and bexarotene were determined previously in F344 rats and Apc\(^{Min}\) mice fed the drugs in an AIN-76A diet (3, 17). We used about 20% MTD as the lower doses of raloxifene and bexarotene in the current efficacy study as described (Fig. 1B). Body weights of animals fed the experimental diets containing raloxifene or bexarotene individually or in combination were comparable with those of animals.
fed the control diet throughout the experimental study, suggesting that the doses of raloxifene, bexarotene or their low-dose combinations did not cause overt toxicity (Fig. 1C).

**Raloxifene, bexarotene and their low-dose combinations reduce colorectal cancer tumor incidence and multiplicity**

The effects of dietary administration of raloxifene, bexarotene and their low-dose combinations on azoxymethane-induced colon tumorigenesis were evaluated. None of the rats in the saline group (without azoxymethane injection, n = 6) developed tumors when autopsied at week 57 weeks of age (data not shown); however, 97.8% of rats administered with azoxymethane developed colorectal tumors. Raloxifene treatment, at both doses, significantly decreased colon tumor multiplicity: at 1.5 ppm, to 3.28 (P < 0.0001) vs control diet; at 3 ppm, to 2.96 (P < 0.0024); to 0.75 (P < 0.007) vs control diet; at 3 ppm (P < 0.0001) vs control diet. Also, both doses of bexarotene significantly inhibited invasive adenocarcinomas (to 1.44 ± 0.21, P < 0.0007 and 1.35 ± 0.19, P < 0.003) compared with control diet (2.15 ± 0.17). A similar reduction in noninvasive adenocarcinomas was observed with low-dose combinations of bexarotene and raloxifene; the reduction was equal to that achieved with high-dose bexarotene. A greater inhibitory effect (61.8%, P < 0.0001) was observed on invasive adenocarcinomas with these low-dose combinations administered during the promotion and progression stages, as compared with high doses of the individual agents alone (Fig. 2B and C).

**Table 1. Expression of ER-β and RXR-α in human colorectal adenocarcinomas by immunohistochemical analysis**

<table>
<thead>
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<th>Percentage staining</th>
<th>Score</th>
<th>Normal-appearing colonic tissues</th>
<th>Colon adenocarcinomas</th>
<th>Normal-appearing colon adenocarcinomas</th>
<th>Colon adenocarcinomas</th>
</tr>
</thead>
<tbody>
<tr>
<td>0%</td>
<td>0</td>
<td>10/12 (83.3%)</td>
<td>1/17 (5.9%)</td>
<td>0/12 (0%)</td>
<td>11/17 (64.7%)</td>
</tr>
<tr>
<td>&gt;0%–5%</td>
<td>1</td>
<td>2/12 (16.7%)</td>
<td>3/17 (17.6%)</td>
<td>1/12 (9.1%)</td>
<td>4/17 (23.5%)</td>
</tr>
<tr>
<td>&gt;5%–50%</td>
<td>2</td>
<td>0/12 (0%)</td>
<td>6/17 (35.3%)</td>
<td>3/12 (25%)</td>
<td>2/17 (11.7%)</td>
</tr>
<tr>
<td>&gt;50%</td>
<td>3</td>
<td>0/12 (0%)</td>
<td>7/17 (41.2%)</td>
<td>8/12 (66.6%)</td>
<td>0/17 (0%)</td>
</tr>
</tbody>
</table>

carcinomas from animals fed control diet. Both doses of raloxifene (1.5 and 3 ppm) reduced the number of invasive adenocarcinomas by about 35% to 53.5% (P < 0.003 to 0.0001; Fig. 2A–C). Like raloxifene, bexarotene insignificantly reduced adenomas (to 0.89 ± 0.15 at 50 ppm; to 0.80 ± 0.13 at 100 ppm) compared with control diet (1.02 ± 0.12). However, bexarotene at both the dose levels caused significant reduction in noninvasive adenocarcinomas (to 0.81 ± 0.12 at 50 ppm, P < 0.0024; to 0.75 ± 0.14 at 100 ppm, P < 0.0002) compared with control diet (1.44 ± 0.13). Also, both doses of bexarotene significantly inhibited invasive adenocarcinomas (to 1.44 ± 0.21, P < 0.0007 and 1.35 ± 0.19, P < 0.003) compared with control (2.15 ± 0.17). A similar reduction in noninvasive adenocarcinomas was observed with low-dose combinations of bexarotene and raloxifene; the reduction was equal to that achieved with high-dose bexarotene. A greater inhibitory effect (61.8%, P < 0.0001) was observed on invasive adenocarcinomas with these low-dose combinations administered during the promotion and progression stages, as compared with high doses of the individual agents alone (Fig. 2B and C).

Histopathologic analysis by hematoxylin and eosin staining revealed that 78% of the colon tumors from the control group were adenocarcinomas, and the remaining 22% were adenomas (Fig. 2A–C). Low- and high-dose raloxifene reduced adenomas and noninvasive adenocarcinomas by up to 23%, although this decrease was statistically insignificant as compared with adenomas and noninvasive adenocarcinomas by about 35% to 53.5% (P < 0.003 to 0.0001; Fig. 2A–C). Like raloxifene, bexarotene insignificantly reduced adenomas (to 0.89 ± 0.15 at 50 ppm; to 0.80 ± 0.13 at 100 ppm) compared with control diet (1.02 ± 0.12). However, bexarotene at both the dose levels caused significant reduction in noninvasive adenocarcinomas (to 0.81 ± 0.12 at 50 ppm, P < 0.0024; to 0.75 ± 0.14 at 100 ppm, P < 0.0002) compared with control diet (1.44 ± 0.13). Also, both doses of bexarotene significantly inhibited invasive adenocarcinomas (to 1.44 ± 0.21, P < 0.0007 and 1.35 ± 0.19, P < 0.003) compared with control (2.15 ± 0.17). A similar reduction in noninvasive adenocarcinomas was observed with low-dose combinations of bexarotene and raloxifene; the reduction was equal to that achieved with high-dose bexarotene. A greater inhibitory effect (61.8%, P < 0.0001) was observed on invasive adenocarcinomas with these low-dose combinations administered during the promotion and progression stages, as compared with high doses of the individual agents alone (Fig. 2B and C).

Because the histologic evaluation revealed that the majority of tumors were adenocarcinomas, the expression of PCNA, a marker for cell proliferation, was determined in the adenocarcinomas from the control and treated groups. The colon tumors from groups fed raloxifene, bexarotene and the combination showed significant reduction of PCNA staining compared with the control group (Fig. 3A). Qualitative microscopic examination of PCNA-stained sections showed a substantial decrease in PCNA-positive cells in tumors from rats exposed to low-dose combinations compared with tumors from rats fed the control diet. Low-dose combinations significantly suppressed proliferation in the colon tumors as compared with control (Fig. 3A). The quantification of PCNA staining showed 55.1% (mean ± SEM) PCNA-positive cells in control tumors, as compared with 35 ± 1.4, 30 ± 2.3, 4.6 (means ± SEM) PCNA-positive cells in tumors from raloxifene-, bexarotene- and their low-dose combination–treated mice, respectively, accounting for a decrease in the proliferation index of about 65% (P < 0.0001) with the combination treatment.
Figure 3C and D summarize the effects of raloxifene, bexarotene and their combinations on tumor cell apoptosis. Qualitative microscopic examination of TUNEL-stained cells showed a substantial increase in TUNEL-positive cells in the tumors of treated rats (Fig. 3C). The quantification of TUNEL-positive cells in polyps from control diet–fed mice showed 6.14 ± 1.40 (mean ± SEM), as compared with 9.6 ± 4.38, 14.4 ± 6.24, and 29.5 ± 6.24 (means ± SEM) positive cells in raloxifene, bexarotene and combination-treated rats, respectively, accounting for an increase in the apoptotic index by >20% (P < 0.0039; Fig. 3D).

Raloxifene, bexarotene and low-dose combinations downregulate expression of β-catenin, cyclin E, cyclin D1, and cdk2 and increase p21 in colon adenocarcinoma

Raloxifene, bexarotene and low-dose combinations of these agents caused significant inhibition of β-catenin protein expression as evident from both immunohistochemistry and Western blotting results (Fig. 3E). Cyclin D1 also was markedly inhibited by the treatment with low-dose combinations (Fig. 3F). Increased expression of p21 and RXR-α was observed in bexarotene- and combination-treated tumors (Fig. 3G and H). Positive staining for ER-β was observed in both the nucleus and cytoplasm of the control group (Fig. 3I). Surprisingly, no ER-β staining was observed in the bexarotene-treated colon tumors; insignificant staining was observed with raloxifene; and no staining was observed in tumors after the low-dose combination treatment (Fig. 3I). The expression of ER-β mRNA (Fig. 4) correlates with the protein expression results in treated colon tumors. Low-dose combinations also caused a decrease in cdk2 protein expression and cyclin E downregulation in tumors treated with the low-dose combination (Fig. 4). Colon tumors from the control group showed stronger staining for cyclin D1 protein and more cyclin D1 mRNA than did those from the treatment groups, with a strikingly a decreased cyclin D1 difference in low-dose combination group (Figs. 3 and 4). A similar result was seen with ER-β mRNA expression and protein expression in the control tumors (Fig. 5).

We also tested the effects of these agents on cytokines IL-6 and -4. Although the individual agents did not have much effect on IL-4, combination treatments caused increased IL-4 mRNA expression (Fig. 5). Treatment with bexarotene and with the low-dose bexarotene and raloxifene combination was effective in reducing mRNA for the inflammatory cytokine IL-6 in colon tumors (Fig. 5).

Discussion

We previously demonstrated that raloxifene and bexarotene prevented azoxymethane-induced ACF in rats and intestinal tumors in APCmin mice (3, 17). Our previous studies showed high expression of ER-β and decreased expression of RXR-α in azoxymethane-induced rat colon tumors. A similar observation was noted in human colon tumor samples (Fig. 1A; refs. 3, 16). Various studies have shown expression of ER in colon cancer cell lines and human colon tissue (5, 19). However, other studies report decreased or no expression of ER in human colon cancer tissue (1, 20, 21), leaving the role of ER in colon cancer
complex. Large randomized placebo-controlled trials have suggested that a combination of estrogen and medroxyprogesterone acetate (MPA) resulted in a 37% reduction in the number of colon cancers compared with placebo at 5 years of follow-up (6, 22). However, analysis of this trial also suggested that women who received hormone replacement therapy had more lymph node involvement with aggressive metastatic tumors compared with those who received placebo (6). However, administration of estrogen alone did not affect significantly the risk of colon cancer development (22, 23). Although few reports suggest protective effect of ER-β in animal models (24–26). In an in vivo study in mice lacking ER-β, administration of azoxymethane or dextran sulfate sodium (DSS) resulted in an increase in colonic tumors (24). In contrast, a recent report by Heijmans and colleagues (27) suggested that estrogen treatment resulted in invasive adenocarcinomas and that the tumor-promoting effects of estrogen were through ER-α and -β. A similar result of increase in tumors with estrogen treatment was observed even in male mice (27). On the basis of the previous reports, raloxifene has been shown to inhibit colon tumor growth in both male and female mice, depending on the endogenous levels of estrogen (28). Although this agent was effective in both the genders, efficacy was more with colon tumors of female mice than...
with male mice (28). These results suggest a positive role of estrogen in inducing tumor growth and development.

The inhibition of ER-β expression with raloxifene administration during promotion and progression stages of colon tumorigenesis may help to inhibit colon tumor formation, specifically to decrease invasive adenocarcinomas (Fig. 2). These observations suggest that raloxifene halted the tumors at the adenoma and noninvasive adenocarcinoma stages and restricted their progression to invasive adenocarcinomas. These results are similar to observation reported in a STAR trial with raloxifene where invasive breast cancer was inhibited with its use (29). Martinez and colleagues reported a 2-fold increased risk for advanced neoplasia in patients who were classified as high risk previously (30). Therefore, the dosing paradigm used in this study can be used for an intervention trial for these high-risk patients to avoid the spread of disease. It is known that estrogen and ER play a role in proliferation of enterocytes and reports suggest that estrogen and ER increase proliferation of epithelial cells (3, 27). Several clinical trials (STAR; ref. 29), MORE (31), and CORE (32) show that raloxifene does not reduce the risk of ER-negative invasive breast cancer. This result suggests that the raloxifene inhibition of invasive cancers is via the modulation of ER signaling and is consistent with our data showing that control colon tumors have increased ER-β expression and that raloxifene is effective in inhibiting these tumors. We observed decreased expression of PCNA in raloxifene-treated tumors, consistent with inhibition of proliferation. Data analysis from the MORE trial suggests that the risk of colorectal cancer may be reduced for those postmenopausal women taking raloxifene (33).

Bexarotene treatment also resulted in inhibition of ER-β expression (Figs. 3 and 4). A similar observation was reported in a mouse model of lung cancer in which bexarotene inhibited progression (34). Bexarotene is the first synthetic nuclear RXR-selective retinoid approved by the FDA for the treatment of refractory cutaneous T-cell lymphoma in all stages; and a biomarker trial with bexarotene in women with high breast cancer risk is recently completed and no data are yet available of this trial (35). We have reported preventive effects of bexarotene against intestinal tumors in APC^{Min} mice (17) and also have been shown to be profoundly effective as both a preventive and a therapeutic agent in a chemically induced mammary cancer model (36). Previous studies had shown that bexarotene was highly effective in preventing these ER+ tumors (37); and it was effective against ER− mammary cancers as well (36, 38). In addition, bexarotene has been shown to have strong synergistic effects with SERMs (39). Thus, suggesting a significant cross-talk between the ER-β and RXR-α. Because raloxifene treatment leads to a decrease in all ER-β isoforms and bexarotene increases RXR-α. A low-dose combination of raloxifene and bexarotene to inhibit colonic tumors based on the mechanistic cross-talk that might provide the synergistic effects. It is noteworthy that low-dose
combination of raloxifene and bexarotene provided about 62% inhibition of invasive adenocarcinomas as compared with about 32% inhibition by individual agents; supporting possible cross-talk between ER-β and RXR-α. Bexarotene did not display a striking dose response in inhibiting colon tumor incidence or tumor multiplicity compared with control, although a trend for dose-associated decrease in colon adenocarcinoma was observed. However, at high dose, bexarotene caused significant suppression of both noninvasive and invasive adenocarcinomas compared with control. Low-dose bexarotene suppressed only invasive adenocarcinomas (Fig. 2). This may be due to the antiproliferative effects of bexarotene on the epithelial cells inhibiting invasive capacity of the malignant cells.

The promotion and progression phases of carcinogenesis are associated with increase in cell proliferation and decrease in apoptosis (40, 41). Here, we observed a decrease in cell proliferation and an increase in apoptosis upon treatment with raloxifene, bexarotene and combinations during the promotion and progression stages (Fig. 3). Because stabilization of β-catenin can lead to colon tumor growth (42), we examined the effect of raloxifene and bexarotene on expression of β-catenin and the downstream signaling molecules cyclin D1 and cyclin E-cdk2. β-Catenin and cyclin D1 were downregulated in response to the individual agents and their combination (Figs. 3–5). Brown and colleagues (43) reported that rexinoids suppress premalignant mammmary epithelial cell proliferation by G1 cell-cycle block. The combination treatment also caused decreased expression of cyclin E-cdk2, suggesting cell-cycle block in the G1-phase, with increase in p21 (Figs. 3–5).

Colorectal tumors are known for overexpression of inflammatory markers. Previous studies have shown a decrease in inflammatory cytokines in response to raloxifene treatment in healthy postmenopausal women (44). One clinical trial suggested decreased expression of IL-4 with raloxifene use in postmenopausal women. However, differential effects of bexarotene were observed on IL-4 levels in patients versus healthy patients. IL-4 was increased in healthy patients, whereas in patients with a high tumor burden of T-cell lymphoma, a 50% decrease was observed (45). Here, we examined the effects of long-term feeding of raloxifene and bexarotene on inflammatory markers in azoxymethane-induced rat colorectal tumors. Our results are in accordance with the earlier reports of decreased IL-4 in tumors (Fig. 5). Similar to results observed in patients, more IL-4 was observed under conditions of decreased tumor burden in animals given the combination treatment compared with untreated animals. However, we previously have reported decreased expression of IL-4 and IL-6 in bexarotene-treated colon tumor samples from ApcMin/+ Mice (17). Recently, raloxifene has been reported to have effects on circulating cytokines such as IL-6; but in other studies, effects of raloxifene on these cytokines have been inconsistent (46–49). Our results indicate that bexarotene is more effective than raloxifene in reducing IL-6 in colorectal tumors.
tumors and that combination of these 2 agents showed a similar effect on IL-6 (Fig. 5).

Overall, our results show that raloxifene inhibited invasive adenocarcinomas, that bexarotene inhibited both non-invasive and invasive adenocarcinomas, and that an improved effect on invasive adenocarcinomas was observed. Importantly, low-dose combination of raloxifene and bexarotene when administered during the promotion and progression stages (adenoma) resulted in significant inhibition of azoxymethane-induced colon adenocarcinomas in F344 rats. The anti-invasive activity of the raloxifene and bexarotene combination may have clinical application; thus, these drugs have potential for chemoprevention of colon cancer in high-risk individuals.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors’ Contributions
Conception and design: N.B. Janakiram, A. Mohammed, V.E. Steele, C.V. Rao
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References
SERM and Retinoid in Chemoprevention of Colorectal Cancer


Chemopreventive Efficacy of Raloxifene, Bexarotene, and Their Combination on the Progression of Chemically Induced Colon Adenomas to Adenocarcinomas in Rats

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