Chemopreventive efficacy of raloxifene, bexarotene and their combination on the progression of chemically-induced colon adenomas to adenocarcinomas in rats

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Running Title: SERM and Rexinoid in chemoprevention of CRC

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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/group) were fed diets containing raloxifene (1.5 or 3 ppm), bexarotene (50 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.
Introduction

Colorectal cancer (CRC) 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 CRC is about 1 in 20. Although 90 percent of the cases of the disease occur in people over the age of 50, studies show that CRC is on the rise in younger patients. Changes in lifestyle, hormone replacement therapies and environmental hormone mimics may be playing a role in this increase in CRC in young adults.

The biological effects of estrogen are mediated by its binding to one of the structurally and functionally distinct estrogen receptor (ER)s (ER-α and ER-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 CRC 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 et al. (6) observed an increased number of positive lymph nodes and more advanced regional and metastatic disease in women who were on hormone treatment compared vs. placebo (76.2 percent vs. 48.5 percent; 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 CRC compared with those taking placebo. Another study reported no statistical difference in CRC 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 CRC (8-9). A recent case-controlled study reported that men with both an estrogen receptor 2 gene ESR2 genetic variant (rs4986938 risk genotype) and high estradiol level had a higher risk for developing CRC (10). A recent report by Simon et al. (11) showed that an estrogen plus progestin group had no reduced CRC mortality; rather, they observed a non-significant increase in CRC deaths compared with placebo (37 vs. 27 deaths, p = 0.320). The selective estrogen receptor modulators (SERMs) exhibit specific ER agonistic and antagonistic activity by binding to ER-α and/or ER-β (12). Of the SERMs, raloxifene has anti-estrogenic 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 (RXRs) and retinoic acid receptors (RARs) are nuclear receptors that mediate the biological effects of retinoids by their involvement in retinoic acid-mediated gene activation. We and others have identified down-regulation 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/+ mice treated with the selective RXR agonist bexarotene and increased RXRα (15), and inhibition of AOM-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 hetero dimers 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 et al. 2002 (17) bexarotene suppress ER-positive breast cancers by synergizing with SERMs through TGF-β pathway in rat models. In a CRC, multiple genes involving various signaling pathways are deregulated. It is noteworthy that colon tumors over-express ER-β and frequently associated with loss of expression of RXRα. Hence developing ER-β antagonist and RXR-α agonist combination is considered to be a rational approach for colon cancer prevention.

US 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 up-regulation of RXRα in association with down-regulation 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. Horseradish peroxidase–conjugated secondary antibodies were from Santa Cruz Biotechnology, CA. A human tissue array for CRC 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 min twice), rehydrated and washed with phosphate-buffered saline (PBS) for 15 min at room temperature. Specimens were treated in a water bath in 0.01 mol/L citrate buffer (pH 6.0) for 30 min at 100°C, slowly cooled to room temperature and washed with PBS for 5 min at room temperature. After quenching endogenous peroxide with 3% hydrogen peroxide in PBS for 10 min at room temperature, the sections were incubated with a blocking solution (supplied with the Zymed kit) for 60 min 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 h. 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 were graded as follows: 0 (no staining); 1 (>0-5%); 2 (> 5-50%); 3 (> 50%).

Animals and experimental diets

Weanling male F344 rats obtained from Harlan Breeding Laboratories (Frederick, MD) 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 semi purified diets were purchased from Bioserv (Frenchtown, NJ) 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%, *DL*-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 the experimental diets was determined periodically in multiple samples taken from the top, middle, and bottom portions of individual diet preparations to verify uniform distribution.

Bioassay: Chemopreventive Efficacy Evaluation

Beginning at 5 weeks of age, all rats were fed the modified American Institute of Nutrition-76A (AIN-76A) diet. At 7 weeks of age, the animals were given subcutaneous injections of azoxymethane (AOM) at a dose of 15 mg/kg body weight or saline as solvent control once weekly for 2 weeks. 9 weeks after the AOM 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 end point and the dose of bexarotene was based on our previous study with Apc⁰ 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 h and transferred to 80% ethanol for histopathological 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 for tumor grade 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 (1:1500), cyclin D1 (1:500), β-catenin (1:500), p21 (1:500), ERβ (1:500) and RXR-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 X 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 (TdT)-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 h) 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 sodium dodecyl sulfate (10% or 15%)-polyacrylamide gel electrophoresis (SDS-PAGE) 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 captured on Ewen Parker Blue sensitive X-ray films were analyzed by densitometry using image quant software. Immunoblotting with tubulin antibody was done to confirm equal protein loading.

**ER-β, β-catenin, Cyclin D1, IL4 and IL6 messenger RNA Expression by Reverse Transcription-Polymerase Chain Reaction**

Total RNA from tumor samples was extracted using Trizol (Ambion, Foster City, CA) 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, Grand Island, NY). Polymerase chain reaction (PCR) was performed for ER-β, β-catenin, Cyclin D1, Interleukin (IL)4 and IL6 using the following conditions: ER-β primers and conditions were as used in the report by Price et al. (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'-CGGGATCCACAAGAAACGGCTTTCA-3' (sense) and 5'-GAGAATTCCAGGTATCAAAACCA-3' (antisense). IL-4 oligonucleotide primer sequences used were as follows: 5'-GCCCCCACCTTGCTGTCACC-3' (sense) and 5'-GCGAAGCACCTGGAAAGCC-3' (antisense) and IL-6 oligonucleotide primer sequences used were as follows: 5'-GACTGATGTTGACAGCCTACTGC-3' (sense) and 5'-TAGCCACTCTTCTCTGACTCTAAT-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 20 seconds, and 72°C for 45 seconds. The oligonucleotide primer sequences used for the cyclin D1 gene were as follows: 5'-CTGGCCATGAACTACCTGGA-3' (sense) and 5'-GTCACACTTGATCACTCTGG-3' (antisense). PCR was done using the Taq polymerase Master Mix (Qiagen, Valencia, CA). The PCR products were visualized and photographed under UV illumination.

**Statistical analysis**

Tumor multiplicity, defined as mean number of tumors/rat, was analyzed by unpaired student “t” test with Welch’s Correction. The tumor incidence (percent of rats with colon tumors) was analyzed by Fisher’s 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 performed using GraphPad Prism Software 5.1 (GraphPad Software, Inc, San Diego, CA).

**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 CRC, we examined expression of the two receptors in a tissue array consisting of samples from patients (male and female from 25 – 79 years of age) diagnosed with adenocarcinoma, grades I to III. 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 upper panel). ER-β showed strong/granular staining in tumor tissue with very weak to no staining in normal tissue (Fig. 1A lower panel). The quantitative analysis of ER-β antibody staining showed a majority of patients (94%) colon adenocarcinomas had over-expression (41% score of 3; 35% score of 2, and 17% scored of 1) as compared to 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 to 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 over-expression of ER-β and down-regulation 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 ApCMin/+ mice fed the drugs in an AIN-76A diet (3, 17). We used ~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 to 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 CRC tumor incidence and multiplicity**

The effects of dietary administration of raloxifene, bexarotene and their low dose combinations on AOM-induced colon tumorigenesis were evaluated. None of the rats in the saline group (without AOM injection, n = 6) developed tumors when autopsied at week ~57 weeks of age (data not shown); however 97.8 % of rats administered with AOM developed colorectal tumors. Raloxifene treatment, at both doses, significantly decreased colon tumor multiplicity: at 1.5 ppm, to 3.28 ± 0.31 (29%, p<0.004); at 3 ppm, to 2.96 ± 0.30 (36%, p<0.0006) (Fig. 1D). A significant reduction in tumor multiplicity was observed with both low dose (33%, 3.14 ± 0.35, p<0.003) and high dose (37%, 2.90 ± 0.28, p<0.0002) bexarotene compared with control diet group. Importantly, low dose combinations of raloxifene and bexarotene caused 47% inhibition ([2.46 ± 0.27, p<0.0001]) of total colon tumors as compared with control diet (Fig. 1D and E). Raloxifene had a modest effect on the incidence of colon tumors compared with control diet fed rats by 5.6 to 11.2 %; but this reduction did not reach statistical significance (Fig. 1E). Similarly, bexarotene at 50 ppm and 100 ppm, did not cause significant reduction in colon tumor incidence (8.4 – 11.2 % decrease as compared with control diet group; Fig. 1E). Thus, neither the low nor high doses of raloxifene or bexarotene caused significant reduction in the colon tumor incidence; but the low dose combination of these two agents caused significant reduction.
(p<0.04) in the colon tumor incidence compared with control diet group (Fig. 1E). Colon tumor multiplicity in rats fed control diet was 4.62 ± 0.38 (mean ± SE).

Histopathological analysis by hematoxylin and eosin staining revealed that 78% of the colon tumors from the control group were adenocarcinomas (AC), and the remaining 22% were adenomas (Fig. 2A, B & C). Low and high dose raloxifene reduced adenomas and non-invasive ACs by up to 23%, although this decrease was statistically insignificant as compared with adenomas and non-invasive ACs from animals fed control diet. Both doses of raloxifene (1.5 and 3 ppm) reduced the number of invasive ACs by ~35%-53.5% (P < 0.003-0.0001) (Fig. 2A, B & 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 non-invasive ACs (to 0.81 ± 0.12 at 50 ppm, p<0.0024; to 0.75 ± 0.14 at 100 ppm, p<0.002) compared with control diet (1.44 ± 0.13). Also, both doses of bexarotene significantly inhibited invasive ACs (to 1.44 ± 0.21, p<0.007 and 1.35 ± 0.19, p<0.003) compared with control (2.15 ± 0.17). A similar reduction in non-invasive ACs 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 ACs with these low dose combinations administered during the promotion and progression stages, as compared with high doses of the individual agents alone (Fig. 2B & C).

**Raloxifene, bexarotene and their low dose combinations decrease cell proliferation and induce apoptosis in colon adenocarcinoma**

Since the histological evaluation revealed that the majority of tumors were ACs, the expression of PCNA, a marker for cell proliferation, was determined in the ACs 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 colonic tumors as compared with control (Fig. 3A). The quantification of PCNA staining showed 55.1 ± 5.4 (mean ± SEM) PCNA-positive cells in control tumors, as compared with 35 ±1.4, 30.2 ±3.4, 19.2 ±2.4, (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 ~65% (P < 0.0001) with the
combination treatment (Fig. 3B). Figure 3C & 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 tunnel-positive cells in polyps from control diet-fed mice showed 6 ± 1.40 (mean ± SEM), as compared with 9.6 ± 4.38, 14.4 ± 6.24, and 29 ± 5.24 (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 down-regulate 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. 3 E). 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 & 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 down-regulation 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 (Fig. 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 IL-4. Although the individual agents did not have much effect on IL4, 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 IL6 in colon tumors (Fig. 5).
Discussion

We previously demonstrated that raloxifene and bexarotene prevented AOM-induced ACF in rats and intestinal tumors in APC<sup>min</sup> mice (3, 17). Our previous studies showed high expression of ER-β and decreased expression of RXR-α in AOM-induced rat colon tumors. A similar observation was noted in human colon tumor samples (Fig. 1A) (3, 16). Various studies have shown expression of ER in colon cancer cell lines and human colon tissue (19, 5). 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 follow-up (22-23). However, analysis of this trial also suggested that women who received hormone replacement therapy had more lymph node involvement with aggressive metastatic tumors as compared with those who received placebo (22). However, administration of estrogen alone did not affect significantly the risk of colon cancer development (23 -24). Whereas few reports suggest protective effect of ER-β in animal models (25 - 27). In an <i>in vivo</i> study in mice lacking ER-β, administration of AOM or dextran sulphate sodium (DSS) resulted in an increase in colonic tumors (25). In contrast, a recent report by Heijmans <i>et al.</i> (28) suggested that estrogen treatment resulted in invasive adenocarcinomas and that the tumor-promoting effects of estrogen were through ER-α and ER-β. A similar result of increase in tumors with estrogen treatment was observed even in male mice (28). Based on 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 (29). Though this agent was effective in both the genders, efficacy was more with colon tumors of female mice compared to male mice (29). 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 non-invasive AC stages and restricted their progression to invasive ACs. These results are similar to observation reported in a STAR trial with raloxifene where invasive breast cancer was inhibited with its use (30). Martinez <i>et al.</i> 2009, reported a twofold increased risk for advanced neoplasia in patients who were classified as high risk previously (31). Therefore the dosing paradigm employed in this study can be employed 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, 28). Several clinical trials (STAR (30), MORE (32), and CORE (33) 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 CRC may be reduced for those post-menopausal women taking raloxifene (34).

Bexarotene treatment also resulted in inhibition of ER-β expression (Fig. 3 and 4). A similar observation was reported in a mouse model of lung cancer in which bexarotene inhibited progression (35). 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 is yet available of this trial (36). We have reported preventive effects of bexarotene against intestinal tumors in APC<sup>Min</sup> 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 (37). Previous studies had shown that bexarotene was highly effective in preventing these ER<sup>+</sup> tumors (38); and it was effective against ER<sup>-</sup> mammary cancers as well (37, 39). In addition, bexarotene has been shown to have strong synergistic effects with SERMs (40). Thus, suggesting a significant cross-talk between the ER-β and RXR-α. Since 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 ~62% inhibition of invasive adenocarcinomas as compared to ~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 AC was observed. However, at high dose, bexarotene caused significant suppression of both non-invasive and invasive adenocarcinomas compared with control. Low dose bexarotene suppressed only invasive adenocarcinomas (Fig. 2). This may be due to the anti-proliferative effects of bexarotene on the epithelial cells inhibiting invasive capacity of the malignant cells. It is note
The promotion and progression phases of carcinogenesis are associated with increase in cell proliferation and decrease in apoptosis (41 - 42). 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). Since stabilization of β-catenin can lead to colon tumor growth (43), we examined the effect of raloxifene and bexarotene on expression of β-catenin and the down-stream signaling molecules cyclin D1 and cyclin E-cdk2. β-catenin and cyclin D1 were down-regulated in response to the individual agents and their combination (Fig. 3, 4 & 5). Brown et al. (44) reported that Rexinoids suppress premalignant mammary 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 (Fig. 3, 4 & 5).

Colorectal tumors are known for over-expression of inflammatory markers. Previous studies have shown a decrease in inflammatory cytokines in response to raloxifene treatment in healthy post-menopausal women (45). One clinical trial suggested decreased expression of IL-4 with raloxifene use in post-menopausal women. However, differential effects of bexarotene were observed on IL-4 levels in patients vs. 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 (46). Here we examined the effects of long-term feeding of raloxifene and bexarotene on inflammatory markers in AOM-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 (47 - 50). Our results indicate that bexarotene is more effective than raloxifene in reducing IL6 in colorectal tumors and that combination of these two agents showed a similar effect on IL6 (Fig. 5).

Overall, our results show that raloxifene inhibited invasive ACs, that bexarotene inhibited both non-invasive and invasive ACs, and that an improved effect on invasive ACs was observed. Importantly, low-dose combination of raloxifene and bexarotene when administered during the promotion and progression stages (adenoma) resulted in significant inhibition of AOM-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.

Acknowledgements

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**Figure legends**

Figure 1: A) Immunohistochemical analysis of ERβ and RXRα expression in normal human colon crypt and adenocarcinoma tissues. Chemopreventive effects of raloxifene, bexarotene, or their low-dose combination on AOM-induced colon tumors in F344 rats. B, Experimental design for the bioassay; C, body weights of animals on experimental vs. control diets; D, total colon tumors; the % inhibition of total tumors relative to the control is given above each bar; E, adenocarcinoma multiplicity (number of tumors/rat); and F, incidence (% rats with colon tumors).

Figure 2: Chemopreventive effects of raloxifene, bexarotene, or combination of low-dose raloxifene plus bexarotene on A, AOM-induced colon adenomas; B, non-invasive adenocarcinomas; and C, invasive adenocarcinomas in male F344 rats.

Figure 3: Immunohistochemical analysis of proliferation and apoptotic markers and of ER-β and RXRα in in AOM-induced colon tumors after treatment of F344 rats with raloxifene, bexarotene or the combination. A, Immunohistochemical staining for PCNA in colon tumors from rats fed control diet or treated with raloxifene, bexarotene or the combination of raloxifene and bexarotene. B, Quantification of proliferation in response to the treatments in A. A significant difference was observed in proliferative index between combination-treated and control group tumors. C, TUNEL assay for apoptotic cells in colon tumors from F344 rats fed control diet or treated with raloxifene, bexarotene or the combination of raloxifene and bexarotene. D, Quantification of apoptotic assay in D. E, Immunohistochemical staining for p21 showing increased expression of p21 in colon tumors from animals given the combination treatment compared with colon tumors from rats fed the control diet. F, Immunohistochemical staining for β-catenin showing decreased expression of β-catenin in colon tumors from rats given the combination treatment compared with those from rats fed control diet. G, Immunohistochemical staining for cyclin D1 showing decreased expression in colon tumors from rats given the combination treatment compared with those fed control diet. H, Immunohistochemical staining for ER-β showing decreased expression in colon tumors from rats given raloxifene, bexarotene or the combination compared with those from rats fed control diet. I, Immunohistochemical staining for RXRα showing increased expression of RXRα in colon tumors from rats given bexarotene and the combination treatment compared those fed control diet.
Figure 4: Modulatory effects of raloxifene, bexarotene and their low-dose combinations on β-catenin signaling and cytokine production in AOM-induced rat colon tumors. Modulatory effects of raloxifene, bexarotene and combinations on β-catenin, cyclin D1, cyclin E, cdk2 and p21 protein expression in colon tumors. The label represents - C, duplicates of control colon tumors; R, duplicates of low dose raloxifene treated colon tumors; B, duplicates of low dose bexarotene treated tumors. Graphs represent the relative protein expression levels of β-catenin, cyclin D1, cyclin E, cdk2, p21, in treated colon tumors compared to control untreated colon tumors. Significant suppression of ER-β, β-catenin, cyclin D1, cyclin E, and cdk2 and increased expression of p21 was observed in colon tumors treated with the combination. Results shown are means ± SEM, unpaired “t” test with Welch’s correction.

Figure 5: Modulatory effects of raloxifene, bexarotene and combinations on β-catenin, cyclin D1, ER-β, IL6 and IL4 mRNA expression in colon tumors in F344 rats. The label represents - C, triplicates of control colon tumors; R, triplicates of low dose raloxifene treated colon tumors; B, triplicates of low dose bexarotene treated tumors. Graphs represent the relative mRNA expression levels of IL4, β-catenin, cyclin D1, IL6, ER-β, in treated colon tumors compared to control untreated colon tumors. A significant suppression of β-catenin, cyclin D1, ERβ and IL6 mRNA was observed in tumors from treated F344 rat. An increased expression of IL4 was observed in colon tumors with combination treatments. Results shown are means ± SEM, unpaired “t” test with Welch’s correction.
References:


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

<table>
<thead>
<tr>
<th>Percentage Staining</th>
<th>Score</th>
<th>ER-β Expression</th>
<th>RXR-α Expression</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Normal appearing Colonic tissues</td>
<td>Colon Adenocarcinomas</td>
</tr>
<tr>
<td>0%</td>
<td>0</td>
<td>10/12 (83.3%)</td>
<td>1/17 (5.9%)</td>
</tr>
<tr>
<td>&gt;0-5%</td>
<td>1</td>
<td>2/12 (16.7%)</td>
<td>3/17 (17.6%)</td>
</tr>
<tr>
<td>&gt;5-50%</td>
<td>2</td>
<td>0/12 (0%)</td>
<td>6/17 (35.3%)</td>
</tr>
<tr>
<td>&gt;50%</td>
<td>3</td>
<td>0/12 (0%)</td>
<td>7/17 (41.2%)</td>
</tr>
</tbody>
</table>
Carcinogen: Azoxymethane (AOM), 15 mg/Kg, body weight. Injected s.c. Raloxifene: 1.5 ppm and 3 ppm; Bexarotene: 50 ppm and 100 ppm. 
Ral + Bex: 1.5 ppm and 50 ppm; Total rats: 225; n=36/45 for each group.
Figure 2

A. Adenomas (Mean ± SEM, N=45/36)

- Control
- Ral-1.5 ppm
- Bex-50 ppm
- Bex-100 ppm

B. Non-invasive adenocarcinomas (Mean ± SEM, N=45/36)

- Control
- Ral-3 ppm
- Bex-50 ppm
- Ral-1.5 ppm + Bex-100 ppm

C. Invasive adenocarcinomas (Mean ± SEM, N=45/36)

- Control
- Ral-3 ppm
- Bex-50 ppm
- Ral-1.5 ppm + Bex-100 ppm

All data points are significant at p<0.002.
Figure 3
Figure 4

(A) Western blot analysis of Cyclin E, ER-β, β-catenin, p21, cdk2, and Tubulin. The blots show protein bands for each protein, with molecular weights indicated:

- Cyclin E: 55kDa, 55KDa, 90kDa, 21kDa, 34kDa, 55kDa
- ER-β: 55KDa
- β-catenin: 90kDa
- p21: 21kDa, 34kDa
- cdk2: Tubulin: 55kDa

(B) Relative protein expression (Cyclin D1) Mean±SEM, n=3

- Control: p<0.01
- Ral: p<0.0001
- Bex: p<0.006
- Ral+Bex: **p<0.0001

(C) Relative protein expression (ERβ) Mean±SEM, n=3

- Control: p<0.04
- Ral: p<0.008
- Bex: **p<0.001
- Ral+Bex: **p<0.0001

(D) Relative protein expression (β-catenin) Mean±SEM, n=3

- Control: ns
- Ral: p<0.003
- Bex: ns
- Ral+Bex: **p<0.003

(E) Relative protein expression (p21) Mean±SEM, n=3

- Control: ns
- Ral: p<0.04
- Bex: **p<0.01

(F) Relative protein expression (cdk2) Mean±SEM, n=3

- Control: ns
- Ral: ns
- Bex: ns
- Ral+Bex: p<0.04
Figure 5

(A) Relative mRNA expression (IL-4) Mean±SEM, n=3

(B) Relative mRNA expression (β-catenin) Mean±SEM, n=3

(C) Relative mRNA expression (IL-4) Mean±SEM, n=3

(D) Relative mRNA expression (Cyclin D1) Mean±SEM, n=3

(E) Relative mRNA expression (IL-6) Mean±SEM, n=3

(F) Relative mRNA expression (ERβ) Mean±SEM, n=3

Legend:
- Control
- Ral
- Bex
- Ral+Bex

Significance:
- p<0.05
- p<0.01
- p<0.001
- p<0.0001

Note: All data shown are from at least three independent experiments.
Chemopreventive efficacy of raloxifene, bexarotene and their combination on the progression of chemically-induced colon adenomas to adenocarcinomas in rats

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