The Combination of Tamoxifen and the Rexinoid LG100268 Prevents ER-Positive and ER-Negative Mammary Tumors in p53-Null Mammary Gland Mice

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Abstract

In pursuit of effective therapeutic agents for the estrogen receptor (ER)-negative breast cancer, we previously showed that bexarotene reduced mammary tumor development by 75% in ErbB2 mice. To further improve the effectiveness of breast cancer prevention, we have now investigated the effects of a combinatorial therapy consisting of two cancer preventive drugs. On the basis of the hypothesis, rexinoid LG100268 plus tamoxifen would more effectively prevent the development of both ER-positive and ER-negative breast cancer. We treated p53-null mammary gland mice with tamoxifen and LG100268, individually and in combination. By 60 weeks of age, vehicle-treated mice developed tumors in 52% of transplanted mammary glands, whereas mice treated with tamoxifen and LG100268 developed tumors in only 13% of transplanted mammary glands. To further define the mechanistic effects of this combinatorial treatment, we investigated the effects of tamoxifen and LG100268 on mammary tissue biomarkers. In mammary tissue harvested before tumor development, the proliferation markers Ki67 and cyclin D1 were significantly reduced in mice treated with the combination therapy. In addition, the rexinoid target genes ABCA1 and ABCG1 were induced in both the rexinoid and combination treatment groups, whereas expression remained constant in tamoxifen group. These results show that tamoxifen-LG100268 combinatorial treatment is more effective in preventing mammary tumors than either agent alone. In addition, these studies have identified relevant tissue biomarkers that can be used to show the effect of these agents on mammary tissue. These results support the development of clinical trials of antiestrogen and rexinoid combinatorial therapy for the prevention of patients with high-risk breast cancer. Cancer Prev Res; 1–8.

Introduction

Breast cancer is the second most common cause of cancer-related death in women in the United States (1). Despite improvements in the early detection and treatment of breast cancer, its annual incidence rate in the United States comprises more than 200,000 new cases and 40,000 deaths (2). These data emphasize the importance of identifying effective preventive agents for breast cancer prevention. Results from recent clinical trials have shown that antiestrogens [including the selective estrogen receptor modulators (SERM), tamoxifen and raloxifene] significantly prevent (≥50%) the development of estrogen receptor (ER)-positive breast cancer (3–7). However, these agents do not prevent the highly aggressive ER-negative breast cancers, which account for one-third of all breast cancer cases in the United States and carry poor prognoses (8). Recently, several novel chemopreventive agents targeting nonendocrine signaling pathways have been tested in preclinical models. Of these, retinoids [retinoid X receptor (RXR)-specific retinoids] have been found to be the most effective agents for the prevention of ER-negative mammary tumors in animal models (9–11).

We have previously shown that the RXR-selective retinoid bexarotene effectively suppresses ER-negative tumor development in MMTV-ErbB2 transgenic mice with minimal toxicity (11). Further studies showed that bexarotene also suppressed the development of preinvasive mammary lesions in MMTV-ErbB2 mice (12). These data suggest that RXR-selective retinoids are promising agents for the prevention of ER-negative breast cancer. Bexarotene has also been shown to reduce mammary tumor development by 75% in p53-null mammary gland mice (13). Continuing our pursuit for more effective chemopreventive agents, we found that the rexinoid LG100268 significantly reduces invasive mammary tumor development in MMTV-ErbB2...
mice and that short-term treatment with LG100268 significantly prevents the development of preinvasive mammary lesions, including both hyperplasia and ductal carcinoma in situ (14). Although bexarotene seems to effectively prevent breast cancer, preclinical studies show multiple toxic effects to be associated with therapeutic application of this agent (15, 16). LG100268, on the other hand, is a more selective rexinoid and has been shown to significantly prevent ER-negative mammary tumor development with minimal toxicity (14). These results suggest that the unilateral prevention of both ER-positive and ER-negative breast cancer may require a combination therapy relying on the individual preventive benefits obtained through treatment with both an antiestrogen agent and a rexinoid. In this study, we investigate the effects of tamoxifen-LG100268 combinatorial treatment in the p53-null mammary tumor model. We hypothesize that the combination of tamoxifen with the rexinoid LG100268 will more effectively prevent the development of ER-positive and ER-negative breast cancers than either administered as a single-agent therapy. To test this hypothesis, we use a p53-null mammary gland mouse model that develops both ER-positive and ER-negative mammary tumors. Our results suggest that the combination of an antiestrogen drug and a rexinoid should be considered for future studies in the prevention of both ER-positive and ER-negative breast cancer in high-risk patients.

Material and Methods

Mice

All donor and recipient mice were bred and maintained at Baylor College of Medicine (Houston, TX). The donor mice were Balb/c p53-null mammary gland, and the recipient mice were Balb/c p53 wild-type (17). All mice were maintained in a conventional mouse facility with room temperature set at 22°C and food and water provided ad libitum. The animal facility is accredited by the American Association of Laboratory Animal Care and all the animal experiments were approved by the Institutional Animal Care and Use Committee (IACUC) at Baylor College of Medicine.

Transplantation

The basic transplantation protocol was previously described by Jerry and colleagues (17). Briefly, 1-mm² fragments of mammary duct from 8- to 9-week-old female Balb/c p53-null mammary gland mice were transplanted into both cleared inguinal fat pads of 3-week-old Balb/c p53 wild-type female mice. This procedure results in successful engraftment in more than 90% of the recipient mice. The transplanted cells took 8 weeks to completely fill the mammary fat pads, therefore, all treatments with chemopreventive agents started at 11 weeks of age to avoid any potential agent-induced effects on cellular capability to grow and fill the fat pads. In all transplantation experiments described, 3 different donors were used for each experiment with equal representation within the different groups. The doses of the 2 chemopreventive agents were chosen based on the results from our previous study (14), and were intentionally chosen to examine the effects of minimal and short exposure time (8–13 weeks) doses of the 2 agents.

In animal experiment 1, there were 3 groups of mice composed of 30 to 40 transplants per group, with treatment occurring at 11 to 24 weeks of host age. Group A, representing the control mice, was implanted with a sham pellet; group B were given a tamoxifen pellet (5.0 mg); and group C were administered the rexinoid LG100268 (50 mg/kg body weight: 5 times/wk).

Animal experiment 2 consisted of 4 groups of mice containing 30 to 34 transplants per group (Fig. 2A). Group A, consisting of control mice, was transplanted with sham pellet (11–19 weeks) and fed cottonseed oil (21–29 weeks) by gastric gavage using a 20-gauge gavage needle in a volume of 0.1 mL; group B mice were given tamoxifen implantations (2.5 mg: 11–19 weeks) and fed cottonseed oil (21–29 weeks); group C were implanted with sham pellet (11–19 weeks) and fed rexinoid LG100268 (50 mg/kg body weight) by oral gavages (21–29 weeks); and group D, the combined therapy group, were treated with a 2.5 mg

Figure 1. Rexinoid LG100268 and tamoxifen treatment inhibits development of mammary gland tumorigenicity in p53 mice. A, treatment scheme: at 3 weeks of age p53-null mammary glands were implanted in recipient mice. Beginning at 11 weeks of age, mice in groups A, B, and C were implanted with a sham pellet (sham, a control), implanted with a tamoxifen (Tam) pellet (5.0 mg), or treated by oral gavage with rexinoid LG100268 (Rex) at 50 mg/kg, respectively. Mice were observed daily for toxicity, and tumor growth was measured biweekly. B, Kaplan-Meier plot representing the percentage of transplants tumor free over time for each treatment group [control (sham), tamoxifen treatment (Tam), and LG100268 treatment (Rex)], P < 0.0001 (log-rank test). C, table shows the percentage of transplants free of tumor and with tumors for each treatment condition at 60 weeks after transplant.
tamoxifen pellet and were fed LG100268 (50 mg/kg body weight). The mice were observed several times a week for any apparent signs of toxicity and were palpated weekly for tumors. All detected tumors were removed and fixed for histologic sectioning upon reaching 5 to 10 mm in the maximal measurable diameter. Mice were sacrificed at 60 weeks after transplantation.

In animal experiment 3, these same treatments were given to 4 groups of mice with slight modifications, that is, the tamoxifen dose was increased from 2.5 to 5 mg (see Fig. 4 for treatment schema). Treatment times were for 13 weeks each, and mammary fat pads were collected 4 hours after the last treatment in each group. Glands from each group were collected at the end of animal experiments 2 and 3 for whole mount preparations enabling the analysis of morphologic development. In addition, epithelial cell pellets were prepared from animal experiment number 3 for use in the biomarker studies.

Mammary epithelial pellet preparation
To prepare mammary epithelial cells from mouse mammary glands, the transplanted glands were harvested from euthanized mice, minced into small pieces, and digested with collagenase and hyaluronidase as previously described (18). Thoroughly digested tissue was centrifuged and washed in PBS. Cell numbers were enumerated, aliquoted into fixed numbers per pellet, and stored at −80°C until being processed for RNA extraction for the use of biomarker assays.

RNA purification from fresh tissue
Total RNA purification was conducted using the RNeasy RNA Isolation Kit (Qiagen, Inc.) as recommended by the supplier. Nucleic acid concentrations of the samples were quantified by measuring the absorbance at both 260 and 280 nm. Typically, 5 to 10 μg RNA was isolated from each sample, and 50 to 100 ng of RNA was used for cDNA and synthesis and quantitative reverse-transcriptase (qRT-PCR) experiments.

cDNA synthesis
cDNA was synthesized using the Random Primer Kit and the SuperScriptII First-Strand cDNA Synthesis System (Invitrogen) as recommended by the supplier. Briefly, random primers, deoxynucleotide triphosphates (dNTP), and DNase-treated RNA were mixed and heated to 65°C for 5 minutes,
then chilled on ice. After the addition of Superscript II reverse transcriptase, the tubes were incubated at 25°C for 10 minutes, then at 42°C for 50 minutes, followed by inactivation of the reaction by heating at 70°C for 15 minutes.

Quantitative reverse-transcriptase PCR

The amount of specific RNA transcripts was assayed by qRT-PCR using gene-specific double fluorescence-labeled probes and an ABI 7900 HT real-time qRT-PCR system (Applied Biosystems). The PCR reaction mixture consisted of 300 nmol/L of each primer, 100 nmol/L probe, 0.025 U/μL taq polymerase, 125 μmol/L each dNTP, 3 mmol/L MgCl2, and 1× taq polymerase buffer. Cycling conditions were 94°C for 1 minute, followed by 40 cycles of 94°C for 12 seconds and 60°C for 30 seconds. All primers and probes were designed with Primer Express Software Version 2.0 software (Applied Biosystems). We used 6-carboxyfluorescein as the 5'0 fluorescent reporter in combination with the 3'0 Black Hole Quencher Double-Dye probe (Eurogentech). Standard curves for the quantification of each transcript and cyclophilin (used for normalization) were generated using a serially diluted solution of synthetic templates. Genome equivalent copies were calculated from the standard curve and normalized to cyclophilin. The ratio between the values obtained provided relative gene expression levels. All reactions were conducted in triplicate, and statistical significance was determined by comparing the means of the triplicate samples using the Student t test.

Immunohistochemical analysis

Tumor samples were fixed in 4% paraformaldehyde and then embedded in paraffin. Tissue sections were subsequently mounted on slides and processed for either hematoxylin–eosin (H&E) staining or immunohistochemical (IHC) staining. The H&E staining was conducted by deparaffinizing 4μm tissue sections in xylene. Rehydration of sections in ethanol and water then took place, followed by 7 minutes incubation in hematoxylin. Samples were destained in running water and fixed in acidified alcohol and ammonia. Finally, slides were incubated in eosin for 2 minutes, rinsed in alcohol and xylene, and mounted for evaluation. Red staining = ERα and blue staining = eosin.

For IHC studies, 4μm tissue sections (from animal experiment 2) were mounted onto slides, which were deparaffinized. The endogenous peroxidase was blocked in 3% hydrogen peroxide buffer. Slides were subsequently rinsed in PBS, and nonspecific binding was blocked with 10% albumin. Samples were incubated with primary antibody anti-ER-α (SC-542, 1:100, Santa Cruz Biotechnology, Inc.), overnight at 4°C. This was followed by incubation with biotinylated antirabbit antibody (1:100) for 30 minutes. Peroxidase activity was visualized using the Vector

Figure 4. Effect of combination treatment on the proliferation marker Ki67 and the cell-cycle marker cyclin D1. A, treatment scheme: at 3 weeks of age, p53-null mammary glands were implanted in recipient mice. From 11 to 24 weeks of age, mice in groups A and C were implanted with a sham pellet (sham, a control), whereas mice in groups B and D were implanted with a tamoxifen (Tam) pellet (5.0 mg). From 24 to 37 weeks of age, mice in groups A and B were fed with cottonseed oil (control), whereas mice in groups C and D were fed with rexinoid LG100268 (Rex) at 50 mg/kg/day. B and C, mammary glands were collected from each group [control (oil), tamoxifen treatment (Tam), LG100268 treatment (sham + Rex), and tamoxifen + LG100268 combination treatment (Tam + Rex)], and enriched mammary epithelial cell pellets were used to purify total RNA. The relative mean mRNA expression level versus the level of cyclophilin mRNA from these pooled samples, as measured by qRT-PCR is plotted. B, Ki67 mRNA expression (P < 0.002). C, cyclin D1 mRNA expression (P < 0.02).
NovoRed Substrate Kit (PK-6101, Vector Laboratories, Inc.) and the AEC Peroxidase Substrate Kit, 3-amino-9-ethylcarbazole (SK-4200, Vector Laboratories, Inc.). Finally, the slides were counterstained with hematoxylin for 30 seconds and mounted with cover slips.

Statistical analysis
Tumor-free survival curves were estimated by the Kaplan–Meier method and statistically evaluated using the generalized Wilcoxon test. Every biomarker result is shown as the mean of 3 samples ± SE. Significance was determined using a Student t test. Differences were considered statistically significant if the P value < 0.05.

Results
Tamoxifen-LG100268 combined treatment inhibits development of mammary tumorigenesis in p53-null mice

Our previous studies indicated that the rexinoids bekarotene and LG100268 partially prevent the development of ER-negative breast cancer in MMTV-ErbB2 mice (9, 14) and in p53-null mammary gland mice (13). To study the chemopreventive effects of a combination of the rexinoid LG100268 with tamoxifen, we chose the p53-null mammary gland mouse model, which develops both ER-positive and ER-negative tumors due to a lack of tumor suppressor p53. Figure 1A shows the experimental schema for our first animal experiment. Mice in group A, representing the control mice, were treated with a sham pellet; group B mice were given a tamoxifen pellet (5.0 mg); and group C were fed rexinoid LG100268. The vehicle control mice treated with the sham pellet developed mammary tumors in 47% of transplanted mammary glands at 60 weeks posttransplantation (Fig. 1B), whereas mice treated with tamoxifen (5 mg pellets) developed mammary tumors in 15% of the transplanted mammary glands, and mice treated with the rexinoid LG100268 (50 mg/kg body weight) developed mammary tumors in 8% of transplanted mammary glands (Fig. 1B). The percentage of transplants free of tumors and with tumors for each treatment condition at 60 weeks after transplant is shown in Fig. 1C.

In experiment 2, we strove to optimize the treatment regimen by lowering both the length of exposure time and dose of the drug. Figure 2A shows the experimental schema for this experiment. Group A mice were treated with a sham pellet and fed cottonseed oil; group B mice were treated with a tamoxifen pellet and fed cottonseed oil; group C mice were treated with a sham pellet and fed LG100268 (50 mg/kg body weight), and group D mice were treated with a tamoxifen pellet and fed LG100268 (50 mg/kg body weight). The results of sequential tamoxifen and rexinoid treatment on ER-positive and ER-negative mammary tumorigenesis in p53-null mice are shown in Fig. 2B. Vehicle control mice treated with the sham pellet and rexinoid developed mammary tumors in 52% of the transplants at 60 weeks posttransplantation. On the other hand, mice treated with tamoxifen (2.5 mg pellets) developed mammary tumors in 42% of the transplanted mammary glands, and mice treated with the rexinoid LG100268 developed tumors in 37% of the transplanted mammary glands. However, those mice exposed to tamoxifen and rexinoid LG100268 sequentially developed mammary tumors in only 13% of the transplanted mammary glands. This therapeutic combination provided a significant preventive effect of the agents given sequentially as compared with either agent given alone (P = 0.014). The percentage of transplants free of tumors and with tumors for each treatment condition at 60 weeks after transplant is shown in Fig. 2C. Histopathologically, there were no discernible differences between tumors of the different treatment groups, and standard ductal carcinomas and carcinomas exhibiting a metaplastic histology were present in all groups.

Tamoxifen-LG100268 combined treatment prevents ER-positive tumor development

The p53-null (–/–) mammary gland mouse model represents both ER-positive (20%–30%) and ER-negative (70%–80%) tumors. To determine the effect of rexinoid LG100268 on the development of ER-positive and ER-negative tumors, we analyzed ER-α expression by immunohistochemistry (from animal experiment 2) in the tumors that did arise (Fig. 3A). Seventeen tumors representing 4 treatment groups were analyzed: (i) the vehicle control group (4 tumors), (ii) the tamoxifen group (5 tumors), (iii) the rexinoid LG100268 group (6 tumors), and (iv) the tamoxifen plus rexinoid LG100268 group (2 tumors). Two of the 4 vehicle-treated control group tumors were ER-positive (using the Allred scoring system, the staining intensity of these 2 tumors showed scores of 2 and 4). One of 6 tumors arising in the rexinoid LG100268-treated group was ER-positive (Fig. 3B). Zero of 5 in the tamoxifen-treated group and 0 of 2 in the tamoxifen plus rexinoid–treated group developed ER-positive tumors. These results suggest that tamoxifen prevents the development of ER-positive tumors in these mice. However, ER-positive tumors were seen in mice treated with the rexinoid alone; thus, while LG100268 does delay tumor development, ER-positive tumors are not totally prevented by treatment with the rexinoid alone.

Effect of tamoxifen and LG100268 on biomarker expression
To further delineate the mechanism(s) of growth suppression induced by the combined treatment of tamoxifen and rexinoid LG100268, a third animal experiment (animal experiment 3) was carried out to analyze and define specific biomarkers for tamoxifen–rexinoid LG100268 combinatory treatment. In the third animal experiment, we increased the dose of tamoxifen to 5 mg and increased the treatment time to 13 weeks each, as the cells for the assays were nontumor mammary cells (see Fig. 4A for the treatment schema). We analyzed the expression of 6 genes after 13 weeks of treatment (as described in Methods). These included the proliferation marker Ki67, Trefoil factor 1 (TFF1; previously named pS2), the cell-cycle marker cyclin D1, ATP-binding cassette transporter A1 (ABCA1), ATP-binding cassette, subfamily G member 1 (ABCG1), and insulin-like...
growth factor–binding protein 6 (IGFBP6). Treatment with tamoxifen alone or in combination with LG100268 resulted in a significant reduction in Ki67 levels; however, treatment with rexinoid LG100268 alone produced no such result (Fig. 4B). Cyclin D1 expression was reduced in mammary glands from mice treated with LG100268 alone or in combination with tamoxifen but not in mice treated with tamoxifen alone (Fig. 4C). The expression of pS2, an ER-α–regulated gene, was significantly reduced by treatment with the combination of tamoxifen and the rexinoid LG100268 (Fig. 5A). The expression of the retinoid-regulated genes ABCA1 and ABCG1 (19, 20) as well as IGFBP6 (21) was significantly increased in the mammary glands from mice treated with either LG100268 alone or in combination with tamoxifen but not in mice treated with tamoxifen alone (Fig. 5B–D).

In addition, we analyzed 3 mammary fat pads from each treatment group. While the control group showed normal ductal branching and lateral buds, those in the treatment group each had impaired ductal outgrowth. The rexinoid-treated group showed reduced size of ductal lumens, reduced branching, and reduced lateral buds. The tamoxifen-alone group showed relatively normal ducts, but had drastically reduced lateral buds, and the combination tamoxifen–rexinoid–treated group showed greatly reduced size of ductal lumen, reduced branching, and virtually no lateral buds (Fig. 6).

Discussion
The experiments presented here describe the effects of 2 chemopreventive agents, tamoxifen and the rexinoid LG100268, administered sequentially at low doses on mammary tumorigenesis in a genetically engineered p53-null mammary gland mouse model. The combination of tamoxifen and LG100268 was more effective in preventing mammary tumorigenesis than independent treatment with either agent. These results are of paramount importance in our continued pursuit of ways to effectively prevent breast cancer. The efficacy inherently associated with short-term (8 weeks), low-dose treatment provides additional support for the combination chemoprevention strategy originally
developed by Sporn and colleagues (22). They showed that a combination of rexinoid LG100268 and selective ER modulators was more effective than either agent administered individually in 2 very different models: the chemical carcinogen-induced rat mammary tumor model and the MMTV-c-Neu mouse mammary tumor model. However, there are 2 key differences between the original protocol of Sporn and colleagues and the protocol we used in this study. First, we used a sequential exposure of the 2 agents, whereas Sporn and colleagues used a nonsequential combination of the 2 agents. Second, we exposed recipient mice for a total of 16 weeks, whereas they exposed recipient mice for 52 to 60 weeks. Our experiments suggest that a relatively short exposure time is capable of providing an effective chemoprevention strategy. In addition, our results showing cancer preventive efficacy with low dose, short-term sequential therapy indicates that these agents can be given for brief sequential periods to limit toxicity.

The kinetics of tumor development within the different treatment groups was also informative. It is now clear that tamoxifen delays the progression of early-stage lesions and that once tamoxifen is withdrawn, lesions develop with the same kinetics associated with untreated mice. In the rexinoid LG100268-treated mice, tumors develop in a biphasic manner, with normal kinetics (sharp slope) displayed in the early and late stages, divided by an alternative kinetic (shallow slope) in the midstages. These results can therefore be interpreted in terms of the stage of progression that is susceptible to the chemopreventive effects of the agents administered in this study. In the mouse mammary gland, tumors develop as a consequence of progression through several stages, from the well-defined preneoplastic stage to the invasive stage. Our results suggest that tamoxifen and LG100268 are more effective at preventing the development of preneoplasias than at preventing the progression of preneoplasias into invasive cancers. That interpretation is supported by earlier results that showed that the rexinoid bexarotene was not effective at preventing the progression of preneoplastic outgrowth line to invasive cancer (13) at the same dose that greatly inhibited the development of mammary tumors in normal mammary cells exposed to the rexinoid. In addition, experiments in which tamoxifen was given at early, middle, or late stages of progression for just 4 weeks showed the greatest preventive effect concomitant with administration of the agent early in tumor development (Medina; unpublished data).

The biomarkers measured in the treated mice seem to be sensitive surrogate markers for the exposure of mammary cells to either tamoxifen or rexinoid LG100268. The combination treatment reduced levels of both Ki67 and cyclin D1 expression in the mammary glands indicating that cell-cycle blockade is one of the mechanisms by which the combination prevents tumor development. In addition, the transporter proteins ABCA1 and ABCG1 are markers of rexinoid treatment, and recently, Schimanski and colleagues showed that ABCA1 is diminished in breast cancer tissues (23). We favor the interpretation that induction of transporter proteins, such as ABCA1 and ABCG1, exerts a preventive effect by an as of yet undiscovered mechanism. Our results indicate that low-dose tamoxifen followed by low-dose rexinoid is an effective chemopreventive regimen for preventing ER-positive and ER-negative mammary tumorigenesis with minimal toxicity. The preventive effect of tamoxifen plus LG100268 is primarily due to the suppression of mammary epithelial cell proliferation in the early stages of mammary tumorigenesis, suppressing the development of premalignant mammary lesions, and ultimately preventing the development of invasive breast cancer. Although LG100268 is quite effective in preventing ER-negative breast cancers in MMTV-ErbB2 mice (14), chemoprevention with tamoxifen plus low-dose rexinoid LG100268 results in more effective prevention of the development of both ER-positive and ER-negative breast cancers in p53-null mammary glands. These results support testing the combination of LG100268 and tamoxifen in other preclinical models of breast cancer. Such studies will support future breast cancer prevention trials testing combinations of rexinoids and antiestrogen drugs.

Disclosure of Potential Conflicts of Interest

P.H. Brown is a consultant/advisory board member in Susan G. Komen for the Cure Scientific Advisory Board. No potential conflicts of interest were disclosed by the other authors.

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References


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