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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Running Title: Combination treatment for breast cancer prevention

Keywords: P53 null mammary gland model, Rexinoid, ER-positive and negative Breast cancer

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Text word count: 5,240
Total number of figures and tables: six:

**ABSTRACT**

In pursuit of effective therapeutic agents for the ER-negative breast cancer, we previously demonstrated 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. Based on 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 while 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, while expression remained constant in tamoxifen group. These results show that tamoxifen-LG100268 combinatorial treatment is more effective at preventing mammary tumors than either agent alone. In addition these studies have identified relevant tissue biomarkers that can be used to demonstrate the effect of these agents on mammary tissue. These results support the development of clinical trials of anti-estrogen and rexinoid combinatorial therapy for the prevention of high risk breast cancer patients.
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 U.S. comprises over 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 anti-estrogens (including the selective estrogen receptor modulators (SERMs), tamoxifen and raloxifene) significantly prevent ($\leq 50\%$) the development of 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 U.S. and carry poor prognoses [8]. Recently, several novel chemopreventive agents targeting non-endocrine signaling pathways have been tested in preclinical models. Of these, rexinoids (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 appears 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 anti-estrogen 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 anti-estrogen 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. 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 8-9-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 over 90% of the recipient mice. The transplanted cells took 8wk to completely fill the mammary fat pads, therefore all treatments with chemopreventive agents started at 11wk of age to avoid any potential agent-induced effects on cellular capability to grow and fill the fat pads. In all transplantation experiments described, three different donors were used for each experiment with equal representation within the different groups. The doses of the two chemopreventive agents were chosen based on results from our previous study [14], and were intentionally chosen to examine the effects of minimal, short exposure time (8-13 wk) doses of the two agents.

In animal experiment 1, there were three groups of mice comprised of 30-40 transplants in each group, with treatment occurring at 11-24wk 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 (50mg/kg body weight) (5X/week).

Animal experiment 2 consisted of four groups of mice containing 30 to 34 transplants per group (Figure 2A). Group A, consisting of control mice, was transplanted with sham pellet (11-19 wk) and fed cottonseed oil (21-29 wk) by gastric gavage using a 20-gauge gavage needle in a volume
of 0.1 mL; group B mice were given tamoxifen implantations (2.5mg) (11-19 wk) and fed cottonseed oil (21-29 wk); group C were implanted with sham pellet (11-19 wk) and fed rexinoid LG100268 (50mg/kg body weight) by oral gavages (21-29 wk); and group D, the combined therapy group, were treated with a 2.5 mg tamoxifen pellet and were fed LG100268 (50mg/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 histological sectioning upon reaching 5-10 mm in the maximal measurable diameter. Mice were sacrificed at 60 wk after transplantation, at which time any apparent mammary tumors were resected. At that time any remaining mammary gland tissue was processed for whole mount preparations.

In animal experiment 3 these same treatments were given to four groups of mice with slight modifications, i.e., the tamoxifen dose was increased from 2.5 to 5 mg. (see Figure 4 for treatment schema). Treatment times were for 13wk each and mammary fat pads were collected at 4hr 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 morphological 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 phosphate-buffered saline (PBS).
Cells 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 performed using the RNaseasy RNA isolation kit (Qiagen, Inc., Valencia, CA) as recommended by the supplier. Nucleic acid concentrations of the samples were quantified by measuring the absorbance at both 260 and 280nm. Typically, 5 μg to 10 μg RNA was isolated from each sample, and 50 ng to 100 ng of RNA was used for cDNA and synthesis and quantitative RT-PCR experiments.

**cDNA synthesis:** cDNA was synthesized using the Random Primer Kit and the SuperScriptII™ First-Strand cDNA Synthesis System (Invitrogen, Carlsbad, CA) as recommended by the supplier. Briefly, random primers, deoxynucleotide triphosphates (dNTPs), 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 min, then at 42°C for 50 min, followed by inactivation of the reaction by heating at 70°C for 15 min.

**Quantitative reverse-transcriptase polymerase chain reaction:** The amount of specific RNA transcripts was assayed by quantitative reverse-transcriptase polymerase chain reaction (qRT-PCR) using gene-specific double fluorescence-labeled probes and an ABI 7900 HT real-time qRT-PCR system (Applied Biosystems, Foster City, CA). The PCR reaction mixture consisted of 300 nM of each primer, 100 nM probe, 0.025 U/μl taq polymerase, 125 μM each dNTP, 3 mM MgCl₂, and 1 × taq polymerase buffer. Cycling conditions were 94°C for 1 min, followed by 40 cycles of 94°C for 12 sec and 60°C for 30 sec. All primers and probes were designed with
Primer Express Software Version 2.0 software (Applied Biosystems, Foster City, CA). We used 6-carboxyfluorescein as the 5′ fluorescent reporter in combination with the 3′ Black Hole Quencher™ Double-Dye probe (Eurogentech, Seraing, Belgium). 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 performed in triplicate and statistical significance was determined by comparing the means of the triplicate samples using the Student’s *t*-test.

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

For IHC studies, 4 μm 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-ERα (SC-542, 1:100, Santa Cruz
Biotechnology, Inc., Santa Cruz, CA), overnight at 4°C. This was followed by incubation with biotinylated anti-rabbit antibody (1:100) for 30 min. Peroxidase activity was visualized using the Vector NovaRED Substrate Kit (PK-6101, Vector Laboratories, Inc., Burlingame, CA) and the AEC Peroxidase Substrate Kit, 3-amino-9-ethylcarbazole (SK-4200, Vector Laboratories, Inc., Burlingame, CA). Finally, the slides were counterstained with hematoxylin for 30 sec 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 three samples ± standard error. Significance was determined using a Student’s 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 bexarotene 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 wk post-transplantation (Figure 1B), while mice treated with tamoxifen (5mg pellets) developed mammary tumors in 15% of the transplanted mammary glands and mice treated with the rexinoid LG100268 (50mg/kg body weight) developed mammary tumors in 8% of transplanted mammary glands (Figure 1B).

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 (50mg/kg body weight) and group D mice were treated with a tamoxifen pellet and fed LG100268 (50mg/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 Figure 2B. Vehicle control mice treated with the sham pellet and rexinoid developed mammary tumors in 52% of the transplants at 60 weeks post-transplantation. On the other hand, mice treated with tamoxifen (2.5mg 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 compared to either agent given alone (P=0.014). 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 ERα expression by immunohistochemistry (from animal experiment 2) in the tumors that did arise (Figure 3A). 17 tumors representing 4 treatment groups were analyzed: (1) the vehicle control group (4 tumors); (2) the tamoxifen group (5 tumors); (3) the rexinoid LG100268 group (6 tumors); and (4) the tamoxifen-plus-rexinoid LG100268 group (2 tumors). 2 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). 1 of 6 tumors arising in the rexinoid LG100268-treated group was ER-positive (Figure 3B). 0 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 performed to analyze and define specific biomarkers for tamoxifen-rexinoid LG100268 combinatorial 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 non-tumor mammary cells (see
Figure 4A for the treatment schema). We analyzed the expression of 6 genes after 13 wk 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, Adenosine triphosphate (ATP)-binding cassette transporter A1 (\( ABCA1 \)), ATP-binding cassette, sub-family 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 (Figure 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 (Figure 4C). The expression of \( pS2 \), an ER\( \alpha \)-regulated gene, was significantly reduced by treatment with the combination of tamoxifen and the rexinoid LG100268 (Figure 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 (Figures 5B, 5C, 5D).

In addition, we analyzed three 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 (Figure 6).
DISCUSSION

The experiments presented here describe the effects of two 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 wk), 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 two very different models: the chemical carcinogen-induced rat mammary tumor model and the MMTV-c-Neu mouse mammary tumor model. However, there are two 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 two agents, whereas Sporn and colleagues used a non-sequential combination of the two agents. Second, we exposed recipient mice for a total of 16 wk, whereas they exposed recipient mice for 52-60 wk. 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
trinoid 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 mid-stages. 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 trinoid bexarotene was not
effective at preventing the progression of an existing p53-null 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 trinoid. Additionally, experiments in which
tamoxifen was given at early, middle, or late stages of progression for just four 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 appear to be sensitive surrogate markers for the
exposure of mammary cells to either tamoxifen or trinoid 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
trinoid 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 like ABCA1 and ABCG1 exerts a preventive effect by an as 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 anti-estrogen drugs.

**Disclosure of Potential Conflicts of Interest**

The authors have declared no conflicts of interest.

**Acknowledgments**

We thank Michelle Savage for her editing of this manuscript.

**Grant Support**

This work was supported by the National Institutes of Health grant R01 CA-078480 (P.H.B.), the Breast Cancer SPORE grant P50 CA-58183 (D.M.), and the National Institutes of Health, NCI, Core Grant CA-016672 (M.D. Anderson Cancer Center)
REFERENCES


FIGURE LEGENDS

Figure 1. Rexinoid LG100268 and tamoxifen treatment inhibits development of mammary gland tumorigenecity 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 bi-weekly. 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 percent of transplants free of tumor and with tumors for each treatment condition at 60 weeks after transplant.

Figure 2. The combination treatment of rexinoid LG100268 and tamoxifen prevents development of mammary gland tumorigenecity in p53 mice. A) Treatment scheme: At 3 weeks of age p53 null mammary glands were implanted in recipient mice. From 11-19 weeks of age, mice in Groups A and C were implanted with a sham pellet (Sham, a control), while mice in Groups B and D were implanted with a tamoxifen (Tam) pellet (2.5 mg). From 21-29 weeks of age, mice in Groups A and B were fed with cottonseed oil (Control), while mice in Groups C and D were fed with rexinoid LG100268 (Rex) at 50 mg/kg/day. Mice were observed daily for toxicity, and tumor growth was measured bi-weekly. B) Kaplan–Meier plot representing the percentage of transplants tumor free over time for each treatment group (control (Sham+Oil), tamoxifen treatment (Tam), LG100268 treatment (Rex), and tamoxifen + LG100268 treatment.
LG100268 and tamoxifen prevents ER positive tumor formation. A) Immunohistochemical staining of ER. Immunohistochemical staining was performed as described in Materials and Methods. B) Staining intensity was measured by Allred score, with positive staining counts and total tumor counts listed by treatment group (control (Sham+Oil), tamoxifen treatment (Tam+Oil), LG100268 treatment (Sham+Rex), and tamoxifen + LG100268 combination treatment (Tam+Rex)) in the table.

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 11-24 weeks of age, mice in Groups A and C were implanted with a sham pellet (Sham, a control), while mice in Groups B and D were implanted with a tamoxifen (Tam) pellet (5.0 mg). From 24-37 weeks of age, mice in Groups A and B were and fed with cottonseed oil (Control), while mice in Groups C and D were fed with rexinoid LG100268 (Rex) at 50 mg/kg/day. B-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 vs. 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 ($P<0.02$).
Figure 5. Characterization of the effect of the rexinoid LG100268 and tamoxifen on the expression of pS2, ABCA1, ABCG1 and IGFBP-6. Comparison of the effects of LG100268 alone (Sham+Rex) or in combination with tamoxifen (Tam+Rex) on the transcript levels of mPS2 measured in p53 null mammary gland mice versus that observed with tamoxifen alone (Tam) or in the control (Oil). Relative molecule numbers of: A) mPS2; B) ABCA1; C) ABCG1; and D) IGFBP6 mRNA, were measured by qRT-PCR normalized to cyclophilin. Statistical significance of the changes was determined by Student’s t test (*p < 0.05).

Figure 6. Whole mount images of p53 null mammary glands. Mammary glands were processed and stained for whole mount after the completion of treatments. Glands were treated with tamoxifen alone (Tam+Oil) and in combination with LG100268 (Tam+Rex). Tamoxifen, particularly in combination with LG100268, shows very sparse ducts and narrow lumens compared to those observed for the control (Sham+Oil). The gland treated with LG100268 alone (Sham+Rex) is intermediate in duct structure compared to both the control and the tamoxifen treated glands.
Figure 1

A: Transplantation at 3 weeks, followed by 11-24 weeks of treatment, leading to tumor formation.

- A Group: Sham pellet
- B Group: Tam pellet 5.0 mg
- C Group: Rex 50 mg/kg

B: Graph showing the percent of transplants tumor-free over weeks after transplantation.

- P-value = 0.0001

C: Table summarizing treatment outcomes.

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Figure 3

(A) Micrographs of mammary gland sections from different treatments:
- Oil + Sham
- Rex + Sham
- Tam + Oil
- Tam + Rex

(B) Summary of number of tumor analyzed and number of ER-positive tumors (Allred score):

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<tr>
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<td>5</td>
<td>0</td>
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Figure 6
Cancer Prevention Research

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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