The Rexinoid LG100268 Prevents the Development of Preinvasive and Invasive Estrogen Receptor–Negative Tumors in MMTV-erbB2 Mice

Yuxin Li, Yun Zhang, Jamal Hill, Qiang Shen, Hee-Tae Kim, Xiaochun Xu, Susan G. Hilsenbeck, Reid P. Bissonnette, William W. Lamph, and Powel H. Brown

Abstract

Purpose: To test whether a novel rexinoid, LG100268, prevents the development of preinvasive and invasive estrogen receptor–negative mammary tumorogenesis in MMTV-erbB2 mice.

Experimental Design: For invasive breast cancer prevention, MMTV-erbB2 mice were treated with daily gastric gavage of vehicle, LG100268 (10 mg/kg), or LG100268 (100 mg/kg) for long term starting at 3 months of age. For preinvasive lesion study, mice were treated with daily gastric gavage of vehicle or LG100268 (100 mg/kg) for 4 months.

Results: Long-term treatment with LG100268 significantly prevented invasive mammary tumor development. Median time (age) to tumor development was delayed from 217 days in vehicle group to 357 days in low-dose group. In high-dose group, only 2 of 20 mice developed tumors after 430 days of treatment. Short-term treatment of LG100268 significantly prevented the development of preinvasive mammary lesions including hyperplasia and ductal carcinoma in situ. The cancer prevention effect was associated with reduced expression of Ki67 and cyclin D1 in mammary glands by >80%.

Conclusion: Rexinoid LG100268 is an effective chemopreventive agent in preventing the development of both malignant and premalignant mammary lesions in MMTV-erbB2 mice.
been developed. It was shown to cooperate with arzoxifene to prevent the development of ER-positive breast cancer in rats (15, 16). This rexinoid is much more specific for retinoid X receptor, with no appreciable binding to retinoic acid receptor proteins (17). Thus, LG100268 is expected to be an efficacious chemopreventive agent that is potentially more tolerable than bexarotene for the prevention of ER-negative breast cancer.

In this study, we have investigated the ability of LG100268 to inhibit the development of invasive and noninvasive ER-negative tumors in MMTV-erbB2 mice. These transgenic mice overexpress the wild-type erbB2 gene in the mammary gland and develop ER-negative mammary carcinomas (18). The course of mammary tumorigenesis in these mice is similar to that of humans, which proceeds from hyperplasia, ductal carcinoma in situ, to invasive breast cancer. The results of this study show that the rexinoid LG100268 effectively prevents ER-negative mammary tumorigenesis in MMTV-erbB2 mice. Additionally, LG100268 prevents the development of premalignant lesions in these mice, supporting its clinical usefulness as a chemopreventive agent. These results suggest that LG100268 would be particularly useful for the prevention of breast cancers in women at high risk for breast cancer.

Materials and Methods

Cell lines and cell growth assay. Normal human mammary epithelial cells (HMEC) were obtained from Clonetics. Mouse mammary epithelial cells were prepared from MMTV-erbB2 transgenic mice. Mouse erbB2 tumor cells were prepared from mammary tumors derived from vehicle-treated MMTV-erbB2 mice. Protocols for mouse mammary cell preparations are described elsewhere (19). HMECs were maintained in mammary epithelial basal medium supplemented with the mammary epithelial growth media kit (Cambrex Corp.). Mouse mammary epithelial cells were maintained in DME/E-12 medium (Invitrogen) supplemented with 2% fetal bovine serum, 10 µg/mL insulin, 5 µg/mL epidermal growth factor, 5 µg/mL linoleic acid, 1 mg/mL bovine serum albumin, and 1% antibiotic/antimycotic solution (Invitrogen). Mouse erbB2 tumor cells and T47D cells were maintained in DMEM (Invitrogen) supplemented with 10% fetal bovine serum. For growth assays, cells were seeded in 96-well plates at 1,000 per well (mouse erbB2 tumor cells) or 2,000 per well (HMECs, T47D, and normal mouse mammary epithelial cells) followed by treatments with 0.1% of DMSO, LGD1069 (1 µmol/L), or LG100268 (1 µmol/L) for up to 16 days. Medium was changed every 2 days. Cell proliferation was measured using the CellTiter 96 Aqueous Non-Radioactive Cell Proliferation Assay (Promega). Statistical analyses were done using one-way ANOVA with the data from day 8.

Transgenic mice and retinoids. Female MMTV-erbB2 transgenic mice were obtained from The Jackson Laboratory. Mice were housed in the...
institutional animal facilities and fed a controlled diet of MIN-76A Purified Diet (Harlan Teklad). Virgin animals were used to avoid confounding effects of hormonal surges during pregnancy. LG100268 and LGD1069 were obtained from Ligand Pharmaceuticals, Inc.

Treatment and data collection. For long-term treatment, mice were randomized into three experimental groups (20 mice in each group) and treated with sesame oil or LG100268 (10 or 100 mg/kg) for 6 days/wk starting at 3 months of age. The mice were allowed to age to 3 months before treatment to avoid affecting normal mammary gland development during puberty. LG100268 was suspended in purified sesame oil (Croda, Inc.) and administered by daily gastric gavage using a 20-gauge gavage needle in a volume of 0.1 mL. The mice were observed daily for apparent signs of toxicity and weights were recorded weekly. Tumor growth was measured twice a week with electronic calipers (Mitutoyo). Tumor volume was calculated as \((\text{width}^2 \times \text{length})/2\). Mice were sacrificed when the largest tumor reached the size of 2,000 mm³ or if the mice did not develop a tumor; they were sacrificed approximately at the age of 20 months. At the time of sacrifice, both tumor and normal mammary tissues were resected. Samples were fixed in 4% neutral buffered formalin (4% formaldehyde, phosphate buffered) overnight and then embedded in paraffin.

For short-term treatment to determine whether LG100268 suppresses the development of premalignant mammary lesions, mice were randomized into two groups, with 27 mice in vehicle group and 29 mice in LG100268 group. Beginning from 3 months of age, mice were treated 6 days/wk with either sesame oil or LG100268 (100 mg/kg) administered by gastric gavage. The mice in both groups were sacrificed after 4 months of treatment (when no tumors were grossly visible). The mammary glands were resected and fixed in 4% buffered formalin overnight followed by paraffin embedding.

Histologic and immunohistochemical analyses. Paraffin-embedded tissues were sectioned at 4 μm and processed for H&E staining for routine histologic assessments. Primary antibodies used for immunohistochemical staining include c-erbB2 (1:50; NeoMarkers), cyclin D1 (RM-9104-R7, NeoMarkers), Ki67 (RM-9106-R7, NeoMarkers), and cleaved caspase-3 (1:100; Cell Signaling Technology, Inc.). Briefly, the slides were deparaffinized and then endogenous peroxidase was blocked in 3% hydrogen peroxide buffer. Samples were incubated with primary antibodies at 4°C overnight and then incubated with biotinylated rabbit anti-mouse antibody (1:100) for 30 min. Peroxidase activity was visualized using Vector NovaRed substrate kit (SK-4800, Vector). The slides were counterstained with hematoxylin for 30 s and then mounted with a coverslip.

Statistical analyses. In the long-term experiment, both tumor-free survival and tumor multiplicity were measured. Tumor-free survival was defined from time of birth to first appearance of a palpable tumor (mass \(\geq 100 \text{ mm}^3\)). Tumor-free survival curves were estimated by the Kaplan-Meier product limit method and compared using the generalized Wilcoxon test. Tumor multiplicity was determined by counting the total number of tumors occurring in each animal up to the time of sacrifice. Multiplicity was summarized as means and SEs and compared by one-way ANOVA. In the short-term experiment, numbers of mammary gland showing preinvasive and invasive lesions were counted and analyzed by Fisher’s exact test. Immunohistochemical staining of Ki67, cyclin D1, and cleaved caspase-3 was compared between vehicle and LG100268 groups by Wilcoxon rank sum test. Correlation between the expressions of Ki67 and cyclin D1 was analyzed by Spearman’s correlation test.

Results

Rexinoid LG100268 and bexarotene inhibit the proliferation of both normal and malignant mammary epithelial cells. Our previous studies indicate that rexinoid bexarotene prevents the development of breast cancer by proliferation inhibition (20). To determine whether LG100268 also represses cell proliferation, we first investigated the suppressive effects of LG100268.
on the growth of normal and malignant breast cells. We prepared mouse mammary epithelial cells from MMTV-erbB2 mice, adenocarcinoma cells from MMTV-erbB2 mammary tumors. For human normal and malignant breast cells, we used HMEC and T47D cells. These cells were treated with DMSO, bexarotene (1 μmol/L), or LG100268 (1 μmol/L). Growth rates were measured as shown in Fig. 1. Both LG100268 and bexarotene inhibited cell growth whereas bexarotene had a more profound effect than LG100268 in HMEC, T47D, and MMTV-erbB2 mouse tumor cells (P < 0.01, one-way ANOVA). In contrast, these two retinoids had similar growth-suppressive efficacy in mouse mammary epithelial cells derived from MMTV-erbB2 mice.

**MMTV-erbB2 transgenic mouse model of ER-negative breast cancer.** To determine the cancer-preventive effect of LG100268, we chose an ER-negative mammary tumorigenesis model that simulates oncogenic events seen in human breast cancer (21). MMTV-erbB2 mice carry the unactivated, wide-type erbB2 proto-oncogene whose expression is targeted to breast tissue by the transcriptional control of the MMTV promoter. These mice develop focal tumors beginning at 8 months of age with a median time of 217 days. At 333 days, all mice in the vehicle group developed focal tumors beginning at 8 months of age. We have previously shown that the tumors arising in these mice are ER negative and erbB2 positive (11). ER and erbB2 expression was not affected by LG100268 treatment (the tumors remained ER negative and erbB2 positive). We also found that ductal carcinoma in situ lesions derived from vehicle-treated mice were also ER negative (data not shown).

**LG100268 prevents the development of mammary carcinomas.** Figure 2A shows the experimental scheme for our long-term treatment experiment. Twenty mice in each experiment group were treated when they were at 3 months of age to allow the full development of mammary gland and to avoid any effect of the retinoid on mammary gland development during puberty. The mice were treated with sesame oil or LG100268 by daily gastric gavage. After 430 days of treatment, all the 20 mice in the vehicle group and the 19 mice in the low-dose group developed tumors that reached the size of 2,000 mm³. One animal in the low-dose group became sick and was sacrificed due to respiratory distress and cachexia before the development of a mammary tumor. On histologic review, it was found to have cancer involving the lungs.

There was also a dramatic reduction of tumor multiplicity (number of tumors per mouse). Vehicle-treated mice had an average of 1.4 ± 0.50 tumors per mouse, compared with 1.05 ± 0.39 tumors per mouse in the low-dose group and 0.10 ± 0.31 tumors per mouse in the high-dose group (P < 0.01, ANOVA). Tumor growth rates were assessed as tumor volume doubling time calculated within a 21-day period from the onset of detectable tumors. The tumor growth rates have no statistical difference among these groups (assessed by one-way ANOVA), suggesting that tumors that developed after LG100268 treatment are resistant to LG100268. There were no observed cutaneous toxicities in mice treated with either dose of LG100268. Common retinoid toxicities (hair loss and skin erythema) were not observed in mice treated with either dose of LG100268. There was no weight loss in mice treated with LG100268 (data not shown).

**LG100268 does not affect expression of the erbB2 transgene.** To determine whether the tumor-suppressive effects of LG100268 were due to down-regulated expression of the erbB2 transgene, we investigated the expression of erbB2 in normal and malignant mammary tissues from vehicle and high-dose groups by immunohistochemical analysis. As shown in Fig. 3A, there is no difference between vehicle-treated and LG100268-treated mice in either normal or malignant tissues, indicating that the cancer-preventive effect of LG100268 is not through down-regulation of the erbB2 transgene. Similar results were also observed in Western blot (data not shown).

Histologic analyses were also examined by H&E staining to determine whether LG100268 affects the morphology of normal and malignant mammary tissues. In normal mammary glands, there were markedly less amounts of mammary epithelial tissues in LG100268-treated mice than in vehicle-treated mice. H&E staining showed no difference in either morphology or nuclear grade between invasive tumors derived from vehicle- and LG100268-treated mice (Fig. 3B).

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**Table 1. Median time to tumor development, tumor multiplicity, and tumor growth rate**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>No. mice</th>
<th>Median time to tumor development (d)</th>
<th>% Mice with tumors at end</th>
<th>No. tumors per mouse*</th>
<th>Tumor volume doubling time (d)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sesame oil (vehicle)</td>
<td>20</td>
<td>217</td>
<td>100</td>
<td>1.40 ± 0.50</td>
<td>9.71</td>
</tr>
<tr>
<td>LG100268 (10 mg/kg)</td>
<td>20</td>
<td>357</td>
<td>95¹</td>
<td>1.05 ± 0.39</td>
<td>10.51</td>
</tr>
<tr>
<td>LG100268 (100 mg/kg)</td>
<td>20</td>
<td>Not reached</td>
<td>10</td>
<td>0.10 ± 0.31</td>
<td>9.01</td>
</tr>
</tbody>
</table>

NOTE: Statistical analyses were done using the generalized Wilcoxon test and one-way ANOVA.

¹Mice that had mammary tumors developed either one or two palpable mammary tumors.
LG100268 prevents the development of premalignant lesions. Like carcinogenesis in humans, mammary tumorigenesis in MMTV-erbB2 transgenic mice is a multistage process that proceeds through hyperplasia, mammary intraepithelial neoplasia (MIN; similar to human ductal carcinoma in situ), and invasive cancer (22). To assess whether LG100268 prevents development of these premalignant lesions, MMTV-erbB2 mice were treated with vehicle (N = 27) or LG100268 (N = 29, 100 mg/kg) for 4 months from the age of 3 to 7 months (Fig. 4A). Normal-appearing mammary glands were then removed and processed for histologic and biomarker analyses. We examined the frequency of hyperplasia, MIN lesion, and cryptic invasive cancer in these mice [we examined one mammary gland per animal (the #4 gland for these studies)]. As shown in Table 2, at age 7 months, 37% (10 of 27) of vehicle-treated mice developed hyperplasia whereas only 10% (3 of 29) of LG100268-treated mice had hyperplasia (P < 0.05). The number of mice showing MIN lesions or cryptic invasive tumors was low in the vehicle group; only three animals had MIN lesions whereas only two animals had invasive breast cancers. It should be noted that most of the animals showing MIN lesions or invasive tumors also had hyperplasias, except one animal that had a MIN lesion without apparent hyperplasia. Because multiple lesions developed in one animal, the

![Fig. 3. Comparison of the erbB2 transgene expression and the histologic features of normal and malignant mammary tissues in vehicle- and LG100268-treated mice.](image)

A. Immunohistochemical staining for erbB2 gene. Both normal and tumor tissues were resected, fixed, and embedded in paraffin. The sections were stained with polyclonal anti-erbB2 antibody. B. Histologic features of normal mammary tissues and mammary tumors. Tissues sections from both vehicle- and LG100268 (100 mg/kg)–treated animals were stained with H&E. Representative fields containing normal and tumor samples.
number of animals showing any lesion is less than the number of individual lesions summed together. In the LG100268-treated group, none of the mice developed MIN lesions or invasive tumors, although this difference did not reach statistical significance.

**LG100268 inhibits proliferation of mammary epithelial cells and reduces expression of cyclin D1.** Proliferation inhibition and apoptosis promotion are two major causes of growth suppression. To determine which mechanism is attributed to LG100268-induced growth suppression, we examined the effect of LG100268 on proliferation and apoptosis by measuring the levels of Ki67 and cleaved caspase-3 in mammary glands harvested after 4 months of treatment. LG100268 dramatically reduced epithelial cell proliferation by 80% ($P < 0.01$, Wilcoxon rank sum test), as determined by Ki67 immunohistochemical staining (Fig. 5B, Table 3). The percentages of cells showing Ki67 positive staining showed large variation among mice within vehicle group (from 80% to 5%). However, the median level was 36.8%. The high level of Ki67 in some samples is likely due to development of hyperplasia in erbB2-expressing mammary cells. No statistical difference was observed in cleaved caspase-3 levels between vehicle and LG100268 treatments (data not shown), suggesting that LG100268 prevents mammary tumorigenesis primarily through proliferation inhibition. We next investigated whether LG100268 affects the expression of cyclin D1, the key regulator of cell cycle progression (23). As determined by immunohistochemical staining, LG100268 reduced cyclin D1 expression by 80% ($P < 0.05$, Wilcoxon rank sum test; Table 3; Fig. 5B). We also observed strong positive correlation between Ki67 and cyclin D1 expression; shown in Fig. 5A are representative immunohistochemistry pictures for Ki67 and cyclin D1 from consecutive sections. The positive correlations were statistically significant in both vehicle and LG100268 groups (assessed by Spearman’s correlation coefficient).

**Discussion**

In this study, we investigated the effect of the highly selective retinoid LG100268 on the development of invasive and preinvasive mammary tissues. Our results show that the retinoid LG100268 prevents the development of ER-negative mammary tumors in MMTV-erbB2 mice and that LG100268 prevents the development of preinvasive lesions such as hyperplasia and MIN. LG100268 reduced Ki67 and cyclin D1 expression in the mammary glands, indicating that the retinoid

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**Table 2. Comparison of the number of mice showing premalignant lesions and invasive cancers**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>No. mice examined*</th>
<th>No. mice showing</th>
<th>Hyperplasia</th>
<th>MIN (ductal carcinoma in situ)</th>
<th>Invasive tumor</th>
<th>Any lesion</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle</td>
<td>27</td>
<td></td>
<td>10</td>
<td>3</td>
<td>2</td>
<td>11</td>
</tr>
<tr>
<td>LG100268</td>
<td>29</td>
<td></td>
<td>3</td>
<td>0</td>
<td>0</td>
<td>3</td>
</tr>
<tr>
<td>$P$</td>
<td></td>
<td></td>
<td>0.019</td>
<td>0.106</td>
<td>0.228</td>
<td>0.009</td>
</tr>
</tbody>
</table>

**NOTE:** $P$ values were obtained with Fisher’s exact tests, generated for each type of lesion individually.

*For this study, we examined one mammary gland in each animal.
acts through cell cycle blockade. This study is the first to show that LG100268 suppresses the development of premalignant mammary lesions, and supports previous studies (24) that show that LG100268 is an effective agent for the prevention of breast cancer.

Our results suggest that the cancer-preventive activity of LG100268 is through growth inhibition during the multistep process of mammary tumorigenesis. This effect could occur from a very early block in the progression pathway (from normal to hyperplasia) or from a global effect at each step throughout tumor progression. We favor the interpretation that LG100268 suppresses ER-negative tumorigenesis at all stages because LG100268 suppresses the growth of normal and malignant breast cells in vitro (Fig. 1) and established breast tumors in vivo if given after the tumors develop, as shown by Suh et al. (16).

However, it should be noted that, in this study, the growth of invasive breast tumors that arose in mice chronically treated with LG100268 was not inhibited by this rexinoid. Such results show that some tumors can develop resistance to the growth-suppressive effects of rexinoids.

When compared with our previous results using bexarotene (11), these studies show that LG100268 more effectively prevents mammary tumorigenesis. At the time when all the control mice developed tumors, only 10% (high dose) and 45% (low dose) of LG100268-treated mice had tumors, compared with 24% (high dose) and 74% (low dose) in bexarotene-treated mice (11). It should be emphasized that the average life span of a normal mouse is ~18 months. The experiments reported here ended at the time when most of the mice were close to the end of their life span. Of the two mice that developed tumors in the high-dose group, one mouse had tumors at the age of 17.3 months, close to the time that the mouse would die even without a tumor. Thus, LG100268 has almost completely blocked tumor development in MMTV-erbB2 mice.

Because apoptosis promotion is another possible mechanism for LG100268-induced growth suppression, we examined the

| Table 3. Quantitative analyses of Ki67 and cyclin D1 expression |
|--------------------|------------------|------------------|
| Wilcoxon rank sum test | Ki67 | Cyclin D1 |
| Vehicle vs LG100268 | <0.001 | 0.012 |
| Correlation (Ki67 vs cyclin D1) | Vehicle | LG100268 |
| Spearman’s correlation coefficient | 0.927 | 0.770 |
| ρ | <0.001 | 0.013 |

NOTE: Data were generated from 10 animals from each experiment group. Percentages of positive stained cells were quantitated by counting at least 500 cells.
apoptosis rate by measuring the expression of cleaved caspase-3 and observed no statistical difference between vehicle and LG100268 treatments. This result is consistent with the published data, in which LG100268 alone has no significant effects in regulating apoptosis in vivo or in vitro in spite of the fact that LG100268 synergizes with arzoxifene to promote apoptosis (15, 16). The antiproliferation effect of LG100268 is very striking because a 90% reduction in Ki67 expression and an 80% reduction in cyclin D1 expression were observed. We previously reported that both bexarotene and LG100268 blocked cell cycle progression from G0-G1 to S phase, decreased the protein levels of cyclin D1, and decreased the phosphorylation status of retinoblasoma in HMEC cells (20). Landis et al. (25) and Yu et al. (26) showed that mice lacking cyclin D1 activity failed to develop lactating mammary glands and were protected from erbB2-induced tumors. All these data suggest that preventive agents such as LG100268 that strongly repress cyclin D1 will be effective chemopreventive agents.

It should be noted that tumors that developed in LG100268-treated mice are resistant to further LG100268 treatment. This is likely the result of selective pressure toward the development of LG100268-resistant cells in chronically treated animals. This suggests that LG100268 alone will not absolutely prevent the development of all breast cancers, and that combination chemoprevention will be required to effectively prevent all breast cancer developments. Recently, Rendi et al. have observed that the combination of a rexinoid, LG100268, and a selective ER modulator, arzoxifene, synergistically prevented the development of ER-positive breast cancer in rat models (15, 16). More importantly, this combination also synergized to prevent the development of ER-negative breast cancer in MMTV-erbB2 mice (20). These investigators also developed an intermittent chemoprevention protocol in which LG100268 and arzoxifene were given for short periods, followed by more prolonged drug-free rest periods. This intermittent protocol effectively prevented the development of breast cancer while minimizing chronic side effects. Such intermittent combination chemoprevention will likely be the most effective and safest way to use rexinoids for cancer prevention.

Our results indicate that the rexinoid LG100268 is an effective chemopreventive agent in preventing ER-negative mammary tumorigenesis with minimal toxicity in preclinical models. The preventive effect of LG100268 is likely due to suppression of mammary epithelial cell proliferation that occurs early during mammary tumorigenesis, suppressing the development of premalignant mammary lesions and ultimately preventing invasive breast cancer. Whereas LG100268 is very effective in preventing ER-negative breast cancers in MMTV-erbB2 mice, combination chemoprevention with a rexinoid and a selective ER modulator will likely be more effective in preventing the development of both ER-positive and ER-negative breast cancers. Our results support the testing of rexinoids and selective ER modulators in high-risk women to determine whether similar cancer-preventive effects are seen in humans.

References

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