

The Retinoid X Receptor-Selective Retinoid, LGD1069, Prevents the Development of Estrogen Receptor-Negative Mammary Tumors in Transgenic Mice¹

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Abstract

Despite the effectiveness of the selective estrogen receptor (ER) modulators in preventing ER-positive breast cancer, chemopreventive agents still need to be developed for the prevention of ER-negative breast cancers. The naturally occurring retinoids are promising agents for the prevention of human cancers but are too toxic for long-term chronic use. We previously demonstrated that the chemopreventive effects of the retinoids could be separated from the toxicity by using an RXR-selective retinoid, LGD1069. The studies described here demonstrate that LGD1069 effectively suppresses ER-negative tumor development in mouse mammary tumor virus-erbB2 transgenic mice with minimal toxicity. These studies suggest that receptor-selective retinoids are promising agents for the prevention of breast cancer and that they may be particularly useful in preventing ER-negative breast cancer.

Introduction

The American Cancer Society estimates that 54,300 new cases and 40,000 deaths from breast cancer will be diagnosed in the United States in 2002. These statistics make breast cancer the second leading cause of cancer death in women and urge the need to further develop methods for the treatment and prevention of breast cancer. Results from the National Surgical Adjuvant Breast and Bowel Project Breast Cancer Prevention Trial (P-1: BCPT) and the more recent multiple outcomes of raloxifene evaluation (MORE) trial demonstrated the effectiveness of SERMs³ such as tamoxifen and raloxifene in preventing breast cancer (1, 2). Despite the effectiveness of these SERMs in reducing the incidence of ER-positive breast cancer, chemopreventive agents still need to be developed for the prevention of ER-negative breast cancers. Retinoids, vitamin A analogues, are promising agents for the treatment and prevention of breast cancer. Binding of the retinoids to the nuclear retinoid receptors, *i.e.*, retinoic acid receptors (RAR) (α , β , and γ) and RXRs (α , β , and γ), leads to regulation of several cellular processes, including growth, differentiation, and apoptosis (3). Several *in vitro* and *in vivo* studies have shown that retinoids can inhibit the growth and invasion of cancer cells (4, 5). Studies by Anzano *et al.* (6) have shown that the naturally occurring retinoid 9cRA prevents the development of NMU-induced mammary tumors in rats, whereas previous work in our laboratory demonstrated that 9cRA suppresses ER-negative mammary tumor

development in the C3(1)-SV40 Tag transgenic mouse model (7, 8). Results from these preclinical studies have led to the use of 9cRA in humans for the treatment and prevention of cancer. However, in human clinical trials, 9cRA has been found to have significant toxicity, including skin changes, liver toxicity, cracking of the lips, and headaches (9). Therefore, receptor-selective retinoids are now being used to separate the chemopreventive efficacy of retinoids from their toxic side effects. We have previously shown that RXR-selective retinoids suppress tumorigenesis with minimal toxicity compared with RAR-selective retinoids, which are highly toxic (10). In this study, we investigated the ability of the RXR-selective retinoid, LGD1069, to inhibit mammary tumorigenesis in a model particularly relevant to human ER-negative breast cancer, the MMTV-erbB2 transgenic mouse model. These mice overexpress the normal cellular erbB2 protein, which induces the development of ER-negative mammary carcinomas that become invasive and eventually metastasize (11). The results of this study demonstrate that an RXR-selective retinoid can significantly prevent ER-negative mammary tumor development with minimal toxicity and support the development of RXR-selective retinoids for the prevention of human breast cancer.

Materials and Methods

Retinoids. The RXR-selective retinoid used in this study, LGD1069 (Targretin), was obtained from Ligand Pharmaceuticals, Inc. (San Diego, CA).

Transgenic Mice. Female MMTV-erbB2 transgenic mice (obtained from The Jackson Laboratory, Bar Harbor, ME) were housed in the institutional animal facilities. Animals were obtained at 10–12 weeks of age and treated 6 days/week from the age of 3 months until the age of 17 months. Virgin animals were used to avoid confounding effects of hormonal surges during pregnancy. Animals were fed a controlled diet of AIN-76A Purified Diet (Harlan Teklad, Madison, WI).

Treatment and Data Collection. Mice were treated with LGD1069 suspended in purified sesame oil (Croda, Inc., Mill Hall, PA) 6 days/week (Fig. 1A). The retinoid was administered by gastric gavage using a 20-gauge gavage needle in a volume of 0.1-ml containing vehicle 10 mg/kg of LGD1069 or 100 mg/kg of LGD1069. Tumor measurements were made twice a week with electronic calipers (Mitutoyo, Utsunomiya, Japan), and tumor volume was determined by multiplying the square of the width by the length and dividing by two. Individual tumor size and tumor location for each animal was recorded. Weights of all mice were recorded weekly. At the time of sacrifice, each tumor was resected and separate portions were (a) processed for histological analysis, (b) explanted into tissue culture to prepare *in vitro* tumor cell lines, or (c) frozen for future use in biomarker studies. These cells were grown in DMEM containing 10% FBS, 1% glutamine, 1% penicillin/streptomycin, and 1% Fungizone.

Histology and Biomarker Analysis. Histology was performed as described previously (7). Briefly, samples were fixed in 10% neutral buffered formalin (10% formaldehyde, phosphate-buffered) overnight and then embedded in paraffin. Tissue sections were then mounted on slides and processed for H&E staining.

Immunohistochemical staining for erbB2 and ER- α was performed using a

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³ The abbreviations used are: SERM, selective estrogen receptor modulator; ER, estrogen receptor; 9cRA, 9-*cis* retinoic acid; RXR, retinoid X receptor; NMU, *N*-nitroso-*N*-methylurea; MMTV, mouse mammary tumor virus; BrdUrd, bromodeoxyuridine; Tag, T-antigen; CDK, cyclin-dependent kinase; RAR, retinoic acid receptor.

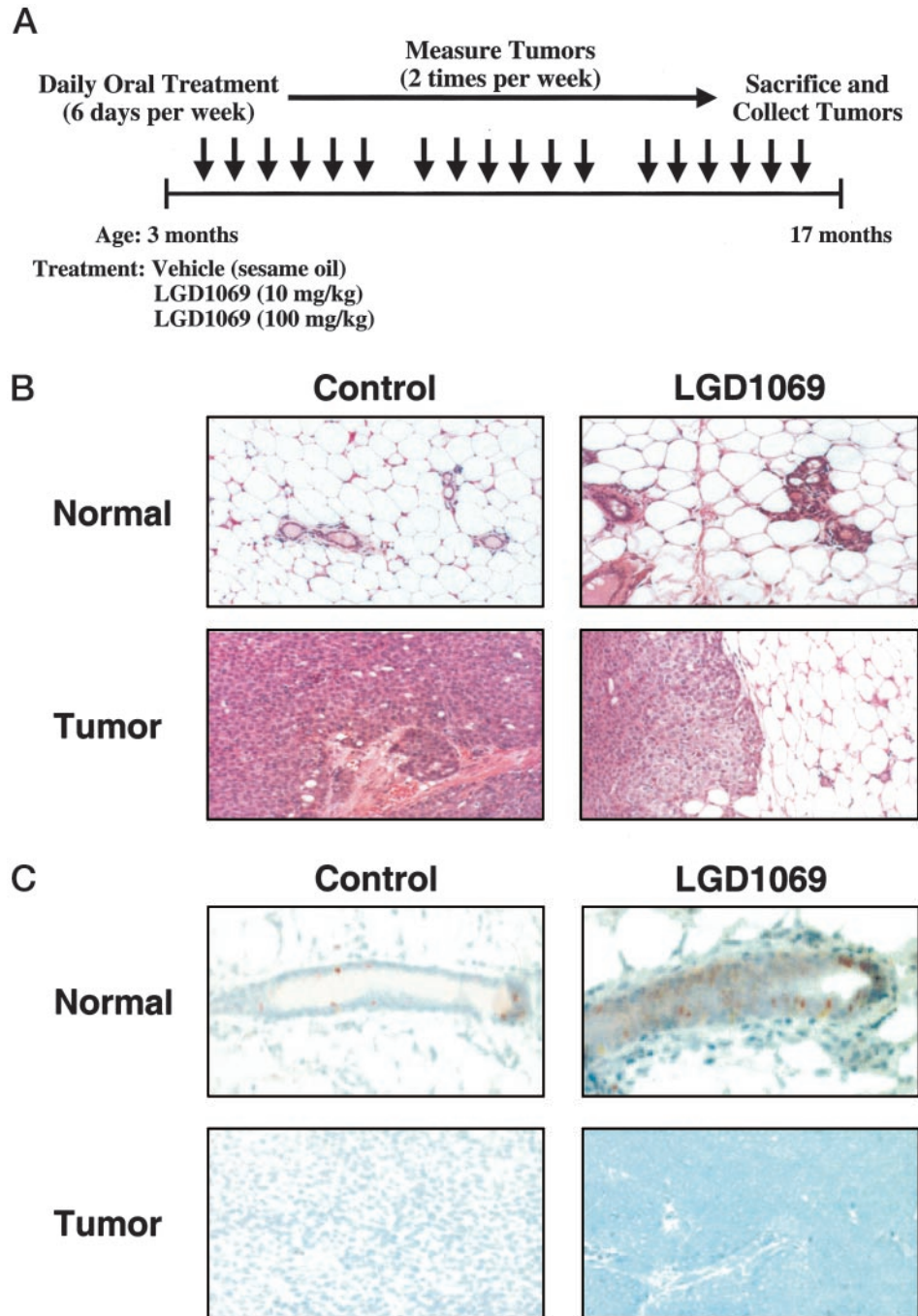


Fig. 1. **A**, Treatment scheme. As described in "Materials and Methods," beginning at 3 months of age, MMTV-erbB2 transgenic mice were treated daily for 6 days/week by oral gavage with either vehicle or LGD1069 (10 mg/kg or 100 mg/kg). Tumor measurements were made biweekly, weights were measured weekly, and symptoms of toxicity were recorded daily. **B**, comparison of histological features of normal mammary tissue and tumors from control and LGD1069-treated mice. At time of sacrifice, tumors were resected, fixed, and embedded in paraffin. Tissue sections were stained using H&E as described in "Materials and Methods." Selected fields containing normal and tumor samples are shown. **C**, expression of ER- α in normal mammary tissue and tumors from control and LGD1069-treated mice. At time of sacrifice, tumors were resected, fixed, and embedded in paraffin. Tissue sections were stained for ER- α as described in "Materials and Methods." Selected fields containing normal and tumor samples are shown.

modified avidin-biotin complex technique as previously described (10) with either a rabbit anti-c-erbB2 polyclonal antibody (1:50; Neomarkers, Fremont, CA) or a rabbit anti-ER- α polyclonal antibody (1:2000; Santa Cruz Biotechnology, Santa Cruz, CA). This was followed by a biotinylated goat antirabbit IgG secondary antibody (H+L; Vector Laboratories, Burlingame, CA).

Staining for BrdUrd was done using the Dako Animal Research Kit (Dako, Copenhagen, Denmark) system. Briefly, tissue sections were cut, mounted onto slides, and deparaffinized. Endogenous peroxidase was blocked with 3% hydrogen peroxide. Slides were then rinsed, and nonspecific binding was blocked (A/B Blocking Kit; Vector Laboratories). BrdUrd was stained using a mouse anti-BrdUrd monoclonal antibody (clone Bu20a; Dako). The slides were then incubated with streptavidin horseradish peroxidase, and peroxidase activity was visualized using 3,3'-diaminobenzidine chromagen intensified with 0.2% osmium tetroxide. Counterstaining was done with Harris Acidified Hematoxylin. The stained sections were reviewed and scored using an ocular grid. The percentage of positive cells was determined for three to five inde-

pendent samples in each treatment group, and results were expressed as an average percentage.

Statistical Analysis of Results. Two outcome measures were considered in this study: tumor-free survival and tumor multiplicity. Tumor-free survival was defined from time of birth to first appearance of a tumor (palpable masses $\geq 100 \text{ mm}^3$). 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 total number of tumors occurring in each animal up to the time of sacrifice. Multiplicity was summarized by means and standard errors and compared by ANOVA.

Results

MMTV-erbB2 Model of Breast Cancer. In this study, we have investigated the ability of the RXR-selective retinoid LGD1069 to inhibit mammary tumorigenesis in the MMTV-erbB2 transgenic

mouse model. As shown in Fig. 1A, MMTV-erbB2 mice were treated with either vehicle or two different doses of LGD1069 from 3 months of age to 17 months of age. The number and size of all mammary tumors were measured twice weekly as described in "Materials and Methods." The mice were observed daily for any apparent signs of toxicity while weights were measured weekly. All mice that did not develop tumors at the end of the experiment are being followed until tumor development occurs. One hundred percent of mice treated with vehicle developed tumors by the age of 17 months (after 416 days of treatment). These mice carry the unactivated neu/c-erbB2 proto-oncogene under the transcriptional control of the MMTV and develop focal tumors (Fig. 1B) beginning at 4 months of age with a median incidence of 205 days. As shown in Fig. 1C, the mammary tumors that develop glands in these mice are ER negative. Normal mammary glands from both the control and treated mice show a few ER-positive cells around the ducts, whereas the tumors are completely negative for ER expression.

LGD1069 Inhibits Development of Mammary Carcinomas.

Fig. 2A shows a plot of the proportion of animals free of tumor *versus* days of treatment. Median time to tumor development for vehicle-treated mice was 234 days. In mice treated with a low dose of LGD1069 (10 mg/kg), the median time to tumor development was significantly delayed to 321 days, and when 100% of the vehicle-treated animals developed tumors, only 74% (17 of 23) of the low-dose-treated mice had developed tumors (Fig. 2B). At the end of the experiment, only 5 mice (24%) treated with high dose of LGD1069 (100 mg/kg) developed tumors. Thus, median time to tumor devel-

opment in the high dose (100 mg/kg) group was not reached and is greater than 416 days of treatment. This delay in time to tumor development was statistically significant ($P < 0.0001$ as assessed by the Generalized Wilcoxon test).

A dramatic effect was also seen on tumor multiplicity (number of tumors/mouse). While vehicle-treated mice developed an average of $1.4 (\pm 0.59)$ tumors/mouse, the low-dose mice developed $0.9 (\pm 0.73)$ tumors/mouse, and the high-dose mice developed $0.21 (\pm 0.41)$ tumors/mouse. This difference in tumor multiplicity between the control and high-dose-treated animals was highly significant ($P \leq 0.001$ as assessed by ANOVA).

There was no weight loss in mice treated with LGD1069. In fact, treatment with LGD1069 caused a slight increase in weight ($< 10\%$). These results demonstrate that the cancer suppressive effect of LGD1069 is not because of general weight loss. None of the mice in the vehicle or low dose of LGD1069 developed any signs of toxicity. Toxicities from high-dose LGD1069 treatment were mild and occurred only after many months (an average of 205 days). These toxicities included hair loss and red ears in up to 46% of the treated animals. These cutaneous toxicities were similar to those seen previously in animals treated with 9cRA (7) but were less severe in these LGD1069-treated animals.

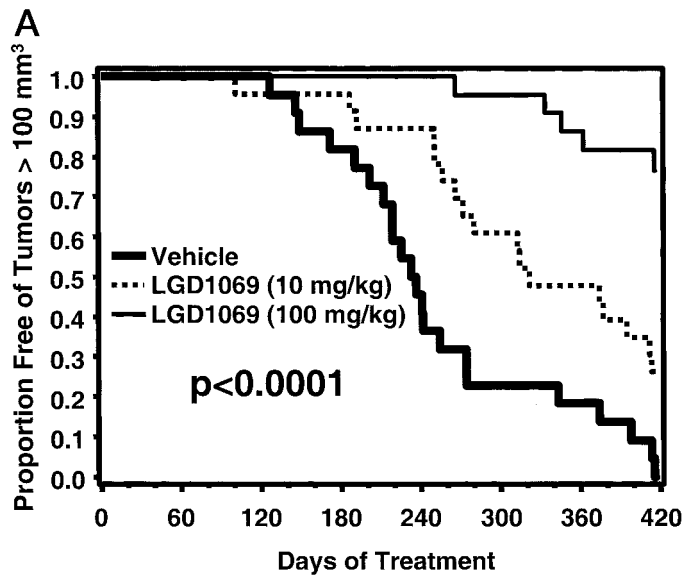
LGD1069 Does Not Affect Transgene Expression. To determine whether the tumor suppressive effects of LGD1069 results were because of down-regulation of the expression of the erbB2 transgene, several analyses were conducted. Immunohistochemistry was performed to detect erbB2 expression in normal and malignant mammary tissue from these mice. As shown in Fig. 3A, the transgene is expressed in a few cells in the normal ducts of both groups of mice. Tumors from both groups express higher levels of erbB2 at similar amounts. Because tumors that arise in animals treated with LGD1069 might still express erbB2, analysis was also conducted on mammary tumor cell lines isolated from control mice and that had never been exposed to retinoids. Levels of erbB2 in these tumor cell lines do not change with LGD1069 treatment (data not shown).

Histological analysis was also conducted using mammary tissue samples from control and LGD1069-treated mice to determine whether LGD1069 affects the morphology of normal or malignant mammary glands in these mice. A representative example of the tumors from vehicle- and LGD1069-treated mice is shown in Fig. 1B. Comparison of tumor samples from vehicle- and LGD1069-treated mice showed no significant difference in morphology or nuclear grade.

LGD1069 Reduces Proliferation in Mammary Tissue. To investigate the mechanism of the tumor suppressive effects of LGD1069, we examined the effect of LGD1069 on proliferation. Prevention of tumor development appears to be attributable to an inhibition of proliferation as shown by a reduction in BrdUrd incorporation. As shown in Fig. 3B, BrdUrd staining is reduced in tumors from LGD1069-treated mice as compared with tumors from vehicle-treated mice. Quantitation of the number of proliferating cells showed that $10.2 \pm 2.0\%$ of the cells from vehicle-treated mice tumors stained positive for BrdUrd, whereas only $4.9 \pm 0.1\%$ of the cells in tumors from LGD1069-treated mice were positive for BrdUrd. Thus, LGD1069 treatment was associated with reduced proliferation in the tumors.

Discussion

The results described here demonstrate that LGD1069 suppresses ER-negative mammary tumor development in MMTV-erbB2 transgenic mice with minimal toxicity. Median time to tumor development was dramatically prolonged in the MMTV-erbB2 mice, and tumor



B

Median Time to Tumor Development and Multiplicity			
Treatment	Median time to tumor development (days)	Percentage of mice with tumors at end	No. of tumors per mouse ^a
Sesame Oil (Control)	234	100%	1.4 ± 0.6
LGD1069 (10 mg/kg)	321	74%	0.9 ± 0.7
LGD1069 (100 mg/kg)	Not reached.	24%	0.2 ± 0.4

^a $p < 0.001$ between groups.

Fig. 2. A, Kaplan-Meier plot of the proportion of animals free of tumor *versus* days of treatment. MMTV-erbB2 mice were treated with either vehicle (sesame oil) or LGD1069 (10 or 100 mg/kg) by gastric gavage from the age of 3 months until the end of the experiment (416 days of treatment). Tumor measurements were made biweekly. Statistical analysis was performed using the Generalized Wilcoxon test. B, median time to tumor development and multiplicity.

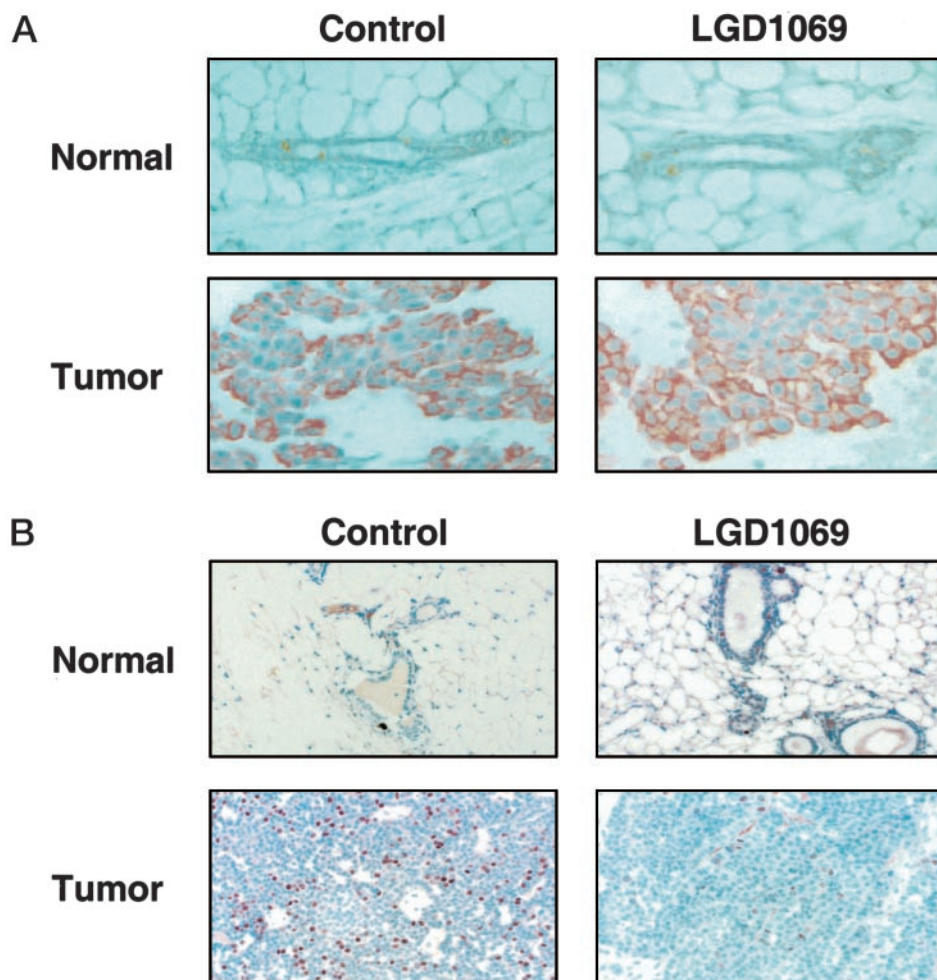


Fig. 3. A, immunohistochemical staining for erbB2. Tumors were resected, fixed, and embedded in paraffin. Tissue sections were prepared for immunohistochemical staining and probed with anti-c-erbB2 polyclonal antibody as described in "Materials and Methods." As shown, no difference in erbB2 expression was observed between vehicle- and LGD1069-treated tumor samples. In normal ducts, both figures demonstrate a few positively staining cells. B, comparison of BrdUrd incorporation in normal mammary tissue and tumors from control and LGD1069-treated mice. At time of sacrifice, tumors were resected, fixed, and embedded in paraffin. Tissue sections were stained as described in "Materials and Methods." Selected fields containing normal and tumor samples are shown. There is an overall reduction in percent positive cells in the LGD1069-treated tumor samples.

multiplicity was decreased. Suppression of tumor development was not attributable to inhibition of transgene expression or induction of mammary cell differentiation. The negative staining of the normal and malignant mammary glands for ER- α suggests that these mice are a good model for ER-negative breast cancer. The data here demonstrate that RXR-selective retinoids, also termed as rexinoids, can indeed suppress the development of ER-negative tumors. Compared with our previous study in the C3(1)-SV40 Tag transgenic mice (10), LGD1069 was even more effective in suppressing tumor development in the MMTV-erbB2 mice. This difference in effectiveness may be attributable, in part, to the mechanisms of transformation: SV40 Tag by inactivation of p53 and retinoblastoma *versus* expression of unactivated erbB2. Retinoids have previously been shown to suppress proliferation by affecting the expression of cyclin D and the activity of CDKs (12). The SV40 Tag causes transformation by inhibiting retinoblastoma and p53 functions. These molecules regulate proliferation at a point distal to the cyclins and CDKs. Given this mechanism, retinoids would be predicted to be less effective at suppressing SV40 Tag-induced tumorigenesis. On the other hand, erbB2 activates signal transduction pathways that activate CDKs. Thus, retinoids would be predicted to interfere with erbB2-induced transformation because the oncogene affects signaling proximal to CDKs.

A previous study by Gottardis *et al.* (13) demonstrated that LGD1069 is able to prevent the development of ER-positive mammary tumors in the NMU-induced rat model. LGD1069-treated rats showed a 90% reduction in both burden and incidence of mammary tumors with no toxicities. Our studies using the MMTV-erbB2 model

demonstrate that LGD1069 can also effectively prevent ER-negative mammary tumor development. Thus, RXR-selective retinoids are likely to be useful for the prevention of both ER-positive and ER-negative breast cancers.

Retinoids have previously been shown to induce differentiation in mammary cells (14), and previous work by Agarwal *et al.* (15) suggests that LGD1069 induces adipocyte differentiation in the mammary gland. In this study, we did not observe any difference in morphology between the vehicle-treated mice and the LGD1069-treated mice, suggesting differentiation. The ability of LGD1069 to prevent tumor development is thus likely predominantly because of its antiproliferative effect. Our experiments here demonstrate that LGD1069 does reduce proliferation in the mammary tumors that develop in these transgenic mice.

Retinoids may be most useful when combined with SERMS to prevent breast cancer development. Studies have already been conducted using retinoids in combination with SERMs such as tamoxifen and raloxifene. Anzano *et al.* (6, 16) demonstrated that 9cRA combined with the SERMs tamoxifen and raloxifene could prevent mammary tumor development in the NMU-induced rat model. More recently, studies by Bischoff *et al.* (17) have shown that a combination of LGD1069 with the SERM tamoxifen had an increased efficacy on inhibiting the growth of NMU-induced mammary tumors. In addition, tumors in their rat model, which had become resistant to tamoxifen, were sensitive to LGD1069 (18). Sporn *et al.* (19) have also recently demonstrated that the combination of an RXR-selective retinoid in

combination with the SERM arzoxifene is even more effective than either agent alone.

On the basis of preclinical studies of LGD1069, this RXR-selective retinoid was tested in human clinical trials. In Phase I clinical trials for the treatment of cancer (20, 21), LGD1069 was found to suppress the growth of cutaneous lymphoma. Additional clinical studies confirmed these effects, and LGD1069 has now been approved for the treatment of cutaneous T-cell lymphoma (22). The first cancer prevention trial using LGD1069 is currently open at our institution. In this trial, LGD1069 is being used as a chemopreventive agent in women at high risk of breast cancer. Thus, the results from this and other studies suggest that LGD1069 will be useful for the prevention of breast cancer and may be particularly effective in combination with SERMs. Additional research into the mechanisms by which retinoids prevent tumor development will help define the role of retinoids in the chemoprevention of breast cancer.

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