Suppression of Mammary Tumorigenesis in Transgenic Mice by the RXR-selective Retinoid, LGD1069

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Abstract
Retinoids have been used in the clinic for the prevention and treatment of human cancers. They regulate several cellular processes including growth, differentiation, and apoptosis. Previously, we demonstrated that a pan-agonist retinoid 9-cis retinoic acid was able to suppress mammary tumorigenesis in the C3(1)-SV40 T-antigen (Tag) transgenic mouse model. However, significant toxicity was seen with this naturally occurring retinoid. We hypothesized that the cancer preventive effects of retinoids could be dissected from the toxic effects by using receptor-selective retinoids. In this study, we used TTNPB, an retinoic acid receptor-selective retinoid, and LGD1069, an retinoid X receptor-selective retinoid, to preferentially activate retinoic acid receptors and retinoid X receptors. In vitro, both compounds were able to inhibit the growth of T47D breast cancer cells. We then determined whether these retinoids prevented mammary tumorigenesis. C3(1)-SV40 Tag mice were treated daily by gastric gavage with vehicle, two different doses of TTNPB (0.3 or 3.0 μg/kg), or two different doses of LGD1069 (10 or 100 mg/kg). Mice were treated from approximately 6–8 weeks to 7–8 months of age. Tumor size and number were measured twice each week, and toxicities were recorded daily. Our data show that LGD1069 suppresses mammary tumorigenesis in C3(1)-SV40 Tag transgenic mice with no observable toxicity, whereas TTNPB had a modest chemopreventive effect, yet was very toxic. Median time to tumor development was 129 days in vehicle-treated mice versus 156 days in mice treated with 100 mg/kg LGD1069 (P = 0.05). In addition, tumor multiplicity was reduced by ~50% in mice treated with LGD1069 (2.9 for vehicle, 2.4 for 10 mg/kg LGD1069, and 1.4 for 100 mg/kg, P = 0.03). TTNPB-treated mice showed a delayed median time to tumor development (131 days for vehicle versus 154 days for 3.0 μg/kg TTNPB; P = 0.05), but no changes were seen in tumor multiplicity. However, toxicity (skin erythema, hair loss) was seen in all of the mice treated with TTNPB. These data demonstrate that receptor-selective retinoids suppress mammary tumorigenesis in transgenic mice and that preventive effects of retinoids can be separated from their toxicity, demonstrating that receptor-selective retinoids are promising agents for the prevention of breast cancer.

Introduction
It is estimated that 193,700 new cases of breast cancer will be diagnosed in the United States in the year 2001, and 40,600 patients will die from their disease (1). Significant improvements in the treatment of breast cancer with chemotherapeutic and hormonal agents have been made over the last 20 years. Additional studies have focused on the prevention of breast cancer. The promising results of recent trials with the antiestrogens tamoxifen and raloxifene (2–4) showed not all breast cancer can be prevented with antiestrogens. Therefore, there remains a need to find other agents for the prevention of breast cancer. Promising agents currently being studied include retinoids, vitamin D analogues, dehydroepiandrosterone derivatives, and monoterpenes (5, 6). Of these, retinoids have been shown previously to suppress cancer development in animals and humans.

Retinoids are vitamin A analogues, which function in regulating cell growth, differentiation, and apoptosis (7, 8). They can bind to the nuclear retinoid receptors, i.e., RARs (α, β, and γ) and RXRs (α, β, and γ; Refs. 9, 10). These receptors bind to specific DNA sequences to regulate gene expression. The RAR and RXR family members are expressed differently during development and differentiation (11), and various isoforms of these proteins can heterodimerize to produce a variety of complexes to regulate different sets of retinoid-induced genes.

In vitro studies have shown that retinoids can inhibit the growth and invasion of cancer cells, and induce them to undergo apoptosis. Several laboratories, including ours, have found many different cancer cell lines to be growth inhibited by...
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9cRA has been shown to inhibit mammary carcinogenesis in the NMU-induced rat model (16), and our previous studies have demonstrated that 9cRA suppresses mammary tumor development in a C3(1)-SV40 Tag transgenic mouse model (17). These results have lead to 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 (18).

We hypothesized that receptor-selective retinoids could be used to separate the chemopreventive efficacy of retinoids from their toxic side effects. Thus, in this study, we have investigated the ability of RAR- and RXR-selective retinoids to inhibit mammary tumorigenesis in the C3(1)-SV40 Tag mouse model, and have compared the side effects seen in animals treated with these retinoids. Our data demonstrate that the RAR-selective retinoid, 9cRA, and the RXR-selective retinoid, LGD1069, suppresses mammary tumorigenesis with no apparent side effects. These results demonstrate that chemopreventive efficacy of retinoids can be separated from toxicity using receptor-selective retinoids. The data support the development of RXR-selective retinoids for the prevention of human breast cancer.

Materials and Methods

Cell Lines and Retinoids. T47D breast cancer cells were obtained from the American Type Culture Collection. Retinoids used in this study, 9cRA (RAR and RXR), TTNPB (RAR-selective), and LGD1069 (Targetretin; RXR-selective) were obtained from Ligand Pharmaceuticals, Inc. (San Diego, CA).

Transient Transfections and Reporter Assays. T47D breast cancer cells were transfected with 1–2 µg of plasmid DNA using FUGENE 6 (Roche). For 6 h, 10−6 M 9cRA, TTNPB, or LGD1069 were added to the cells followed by lysing the cells with a buffer containing 1 mM DTT, 100 mM potassium phosphate (pH 7.8), and 1% Triton X-100. Luciferase activity was assayed according to the luciferase assay kit protocol (Tropix, Inc., Bedford, MA). β-Gal activity was determined by adding 20 µl of either diluted or undiluted cell lysate to 80 µl of β-gal reagent buffer containing 88 mM phosphate buffer (pH 7.3), 11 mM KCl, 1 mM MgCl2, 54.7 mM β-mercaptoethanol, and 4.4 mM chlorophenol red-β-D-galactopyranoside (Roche) at 37°C, and the absorption at 600 nm was determined. Luciferase results were then normalized using the β-gal assay results to control for transfection efficiency.

Growth Curve of T47D Breast Cancer Cells. T47D breast cancer cells were treated with either 0.1% DMSO (control), 9cRA, TTNPB, or LGD1069 (retinoids at 10−6 M) for 8 days. Relative cell growth was measured using the CellTiter 96 Aqueous Non-Radioactive Cell Proliferation Assay (Promega, Madison, WI). As described in the protocol, cells were incubated with 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium and phenazine methosulfate, and conversion into a formazan product was determined at 550 nm. Measurements of cell growth and medium changes were performed every other day.

Transgenic Mice. Female C3(1)-SV40 Tag transgenic mice (obtained from the Animal Production Program, National Cancer Institute, Frederick, MD) were housed in the university animal facilities. These mice have been shown to develop mammary tumors by 6 months of age (19). Animals were obtained at 6–8 weeks of age and treated with retinoids daily for 7 days a week until the age of 7–8 months. Virgin animals were used because the development of mammary tumors does not depend on pregnancy. Animals were fed a controlled diet of Teklad LM-485 Mouse/Rat Diet (Harlan Teklad, Madison, WI).

Treatment and Data Collection. TTNPB and LGD1069 were suspended in purified sesame oil (Croda, Inc., Mill Hall, PA). The retinoids were administered daily for 7 days/week by gastric gavage using a 20-gauge gavage needle in a volume of 0.1 ml containing sesame oil (vehicle), or 0.3 or 3 µg/kg TTNPB, or 10 or 100 mg/kg LGD1069. TTNPB high-dose (3 µg/kg) and low-dose (0.3 µg/kg) mice were treated from 8 weeks of age until sacrifice (up to 25 weeks). The TTNPB low-dose (0.3 µg/kg) mice were treated inadvertently with 30 µg/kg for 1 week, but treatment was changed to 0.3 µg/kg for the remainder of the experiment (weeks 9–25). LGD1069 low-dose (10 mg/kg) mice were treated from 6 to 7 weeks until sacrifice. Because of their small size when young, the mice on high dose of LGD1069 (100 mg/kg) were initially treated with 50 mg/kg and then changed to a 100 mg/kg dose at 7 weeks of age. Tumor measurements were made 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 of the mice were recorded weekly.

At the time of sacrifice, each tumor was resected, and separate portions were: (a) processed for histological analysis; or (b) explanted into tissue culture to prepare in vitro tumor cell lines. These cells were grown in DMEM containing 10% FBS, 1% glutamine, 1% penicillin/streptomycin, and 1% Fungizone (Invitrogen, Carlsbad, CA).

Biomarkers and Histological Analysis. Histology was performed as described previously (17). 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.

The immunohistochemical analysis of phospho-histone H3 (mitosis marker) was performed using a modified avidin-biotin complex technique as described in a previous study (20). Briefly, tissue sections were deparaffinized in xylene and rehydrated in a series of ethanol solutions (100% to 50%). The sections were then microwaved for 15 min to retrieve antigens in 0.01 M citric acid solution. The endogenous peroxidase activity was blocked by incubation in a 1% methanolic hydrogen peroxide solution for 30 min. This was followed by preincubation with 20% normal goat serum to minimize nonspe-
cific binding of the second antibody. The sections were then incubated at 23°C for 4 h with polyclonal mouse antiphospho-histone H3 antibody from Upstate Biotechnology (Lake Placid, NY) diluted at 1:50 in PBS. After being washed three times in PBS, the sections were incubated with biotinylated goat anti-rabbit IgG (H+L; Vector) for 30 min at 23°C and then incubated with the ABC kit (Vector Laboratories, Burlingame, CA) for 30 min in the dark. This was followed by incubation with 3-amino-9-ethylcarbazole (Sigma Chemical Co., St. Louis, MO) solution for 10 min to visualize the peroxidase complex. The sections were finally mounted with Aqua mount medium under coverslips. Control sections were incubated with normal goat IgG instead of primary antibodies or with the second antibody only.

The stained sections were reviewed and scored using an ocular grid. The percentage of positive cells was determined for five independent samples in each treatment group, and results were expressed as an average percentage.

**Plasma Concentrations of Retinoids.** Concentration of retinoids was determined as described previously (17). Briefly, blood was collected in a heparinized tube, and plasma was...
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**Activation of RAR and RXR Reporter Constructs.** To demonstrate the specificity of the receptor-selective retinoids, we transfected luciferase reporter constructs containing retinoic acid response elements specific for either the RAR receptor (a retinoic acid response element, the DR5 element from the retinoic acid response elements specific for either the RAR receptor (21, 22). TTNPB has been shown previously to be specific for the RXR receptor (21, 22); and (b) TTNPB, which binds all RAR isotypes (21); and (c) LGD1069 (100 mg/kg), which binds all RXR isotypes (22). TTNPB has been shown previously to be specific for the RAR receptor, whereas LGD1069 shows specificity for the RXR receptor (21, 22).

**Results**

**Retinoids.** Fig. 1 shows the structure of the retinoids used in this study. Previously the pan-agonist 9cRA (activates both RAR and RXR) was used (17). In this study, two synthetic receptor-selective retinoids were used: (a) TTNPB, which binds all RAR isotypes (21); and (b) LGD1069, which binds all RXR isotypes (22). TTNPB has been shown previously to be specific for the RAR receptor, whereas LGD1069 shows specificity for the RXR receptor (21, 22).

**Inhibition of T47D Breast Cancer Cell Growth.** To demonstrate the growth inhibitory effects of the receptor-selective retinoids on breast cancer cells, we treated T47D breast cancer cells with these retinoids. As shown in Fig. 3, the T47D cells were sensitive to all of these retinoids at different grades, with 9cRA causing the greatest growth inhibition. These results show that T47D breast cancer cells are sensitive to the pan-agonist 9cRA as well as the receptor-selective retinoids TTNPB and LGD1069.

**Table 1** Median time to tumor development and multiplicity

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<th>Treatment</th>
<th>Median time to tumor development (days)</th>
<th>No. of tumors per mouse</th>
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<tbody>
<tr>
<td>Sesame Oil (Control)</td>
<td>131</td>
<td>3.4 ± 0.27</td>
</tr>
<tr>
<td>TTNPB (0.3 μg/kg)</td>
<td>142</td>
<td>3.2 ± 0.22</td>
</tr>
<tr>
<td>TTNPB (3.0 μg/kg)</td>
<td>154</td>
<td>3.3 ± 0.33</td>
</tr>
<tr>
<td>Sesame Oil (Control)</td>
<td>129</td>
<td>2.9 ± 0.44</td>
</tr>
<tr>
<td>LGD1069 (10 mg/kg)</td>
<td>131</td>
<td>2.4 ± 0.40</td>
</tr>
<tr>
<td>LGD1069 (100 mg/kg)</td>
<td>156</td>
<td>1.4 ± 0.29*</td>
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</table>

*P ≤ 0.03 between control and LGD1069 (100 mg/kg) group.

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 ≥100 mm³). 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.

**Inhibition of Tumor Development by LGD1069.** To determine whether RAR- and RXR-selective retinoids can inhibit tumor development in the C3(1)-SV40 Tag mice, we treated mice daily with vehicle or two doses of TTNPB (0.3 or 3.0 μg/kg), or LGD1069 (10 or 100 mg/kg). To measure the efficacy of the different retinoids, the number and size of all of the mammary tumors were measured twice weekly as described in “Materials and Methods.” The mice were also observed daily for any apparent signs of toxicity. All of the animals eventually developed tumors by 200 days of age.

**Table 2** Toxicities in mice treated with TTNPB and LGD1069

<table>
<thead>
<tr>
<th>Toxicity</th>
<th>TTNPB (3.0 μg/kg)</th>
<th>LGD1069 (100 mg/kg)</th>
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<tr>
<td>Skin erythema</td>
<td>100% (15/15)</td>
<td>0% (0/17)</td>
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<tr>
<td>Hair loss</td>
<td>100% (15/15)</td>
<td>0% (0/17)</td>
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* Similar toxicities seen in 9-cis retinoic acid-treated mice.

As shown in Fig. 4 and in Table 1, in mice treated with TTNPB, median time to tumor development was prolonged in the TTNPB-treated mice as compared with vehicle-treated mice (131 days for vehicle versus 142 days for 0.3 μg/kg TTNPB and 154 days for 3.0 μg/kg TTNPB; P ≤ 0.05). In mice treated with low dose of LGD1069 (10 mg/kg), both the control and LGD1069-treated mice showed a similar time to tumor development (~130 days). However, mice treated with 100 mg/kg dose of LGD069 showed a significant delay in tumor development (156 days; P = 0.05 by the generalized Wilcoxon test).

When tumor multiplicity was examined, a more dramatic effect was seen in the LGD1069-treated mice (Table 1). Specifically, the control mice developed an average of 2.9 tumors/mouse, whereas the LGD1069-treated mice developed 2.4 tumors in the 10 mg/kg LGD1069 group and 1.4 tumors in the 100 mg/kg LGD1069 group. This difference in tumor multiplicity between the control and high dose-treated animals was highly statistically significant (P ≤ 0.03 as assessed by ANOVA), and is similar to 9cRA, which showed a 50% reduction in tumor multiplicity (17). On the other hand, in the mice treated with TTNPB, there was no significant change on tumor multiplicity. The control mice as well as both treatment groups showed a similar number of tumors per mouse (approximately three tumors per mouse; P ≤ 0.91).

The toxicity of these retinoids is shown in Table 2. All of the mice treated with the high dose of TTNPB showed skin erythema and hair loss, whereas no toxicities were seen in mice treated with LGD1069. The toxicities seen with TTNPB were similar to those seen with treatment with 9cRA. In addition,
weight loss was observed in TTNPB-treated mice. In mice treated with LGD1069, no observable cutaneous toxicities or weight loss were observed. These results suggest that the RAR-selective retinoid, TTNPB, is highly toxic, whereas the RXR-selective retinoid, LGD1069, is nontoxic.

**Biomarkers and Histology of Tumors.** Histological analysis was conducted using tissue samples from control and retinoid-treated mice to determine whether morphology of mammary glands was affected in these mice. Representative examples of the tumors from vehicle- and retinoid-treated mice are shown in Fig. 5. Comparison of tumor samples showed no significant difference in morphology or nuclear grade. These results suggest that treatment with receptor-selective retinoids do not induce differentiation of the tumor cells that arise in treated animals. Staining for the mitosis marker phosphorylated-histone H3 shows that LGD1069 inhibited phosphorylated-histone H3 expression, whereas TTNPB did not (Fig. 5, 5.9% for vehicle versus 5.2% for TTNPB and 5.7% for vehicle versus 3.8% for LGD1069). This result is consistent with the inhibition of tumor formation and tumor multiplicity induced by LGD1069.

**Plasma Levels of Retinoids.** To determine the plasma levels of the retinoids, we performed pharmacokinetic analysis after acute and chronic dosing in these animals. At time of sacrifice, blood was collected and plasma was isolated as described in “Materials and Methods.” Plasma samples were analyzed using reverse-phase high performance liquid chromatography to determine the plasma concentration of the retinoids.
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The ability of RXR receptor-selective retinoids, particularly the RXR-selective retinoid LGD1069, to suppress tumor development without toxicity in this and other animal models demonstrates that RXR-selective retinoids are promising agents for the prevention of cancer. Retinoids may be most useful when combined with antiestrogens to prevent breast cancer development. Studies have already been conducted using retinoids in combination with antiestrogens such as tamoxifen and raloxifene and have shown when combined that these agents may have increased ability to prevent cancer (16, 24–27). More

Discussion

The results here demonstrate that the RXR-selective retinoid LGD1069 is more effective at suppressing mammary tumor development and is less toxic than the RAR-selective retinoid TTNPB. Previous studies by our laboratory demonstrated that the pan-agonist 9cRA was able to suppress tumor development in the C3(1)-SV40 Tag model, but that 9cRA induced cutaneous toxicities including skin erythema of the ears (17). In this study, we were able to separate the toxicity from the cancer preventive effects of the retinoids using RXR-selective retinoids. Treatment with the RAR-selective retinoid, TTNPB, had a modest effect on tumor development but was quite toxic. TTNPB-induced toxicity included the typical retinoid toxicities of skin erythema, hair loss, and weight loss. On the other hand, LGD1069 suppressed mammary tumorigenesis without toxicity. The chemopreventive effect of LGD1069 was similar to that seen with 9cRA in our previous study (17) suggesting that the cancer preventive activity of 9-cis retinoic acid is mediated through activation of the RXR receptor, whereas the cutaneous toxic effects of retinoids are mediated through activation of the RAR receptor. Studies are currently ongoing to identify the relevant dimerization partners of RXR that mediate the preventive effect of LGD1069.

Studies of the C3(1)-SV40 transgenic mice suggest that tumors arising in these mice are less dependent on estrogen (19). The invasive carcinomas lose ER-α expression early during tumor progression and appear to be hormone independent (19). Thus, these mice may serve as a model for ER-negative breast cancer. Our data with 9cRA (17) and the data here with the receptor-selective retinoids demonstrate that retinoids can suppress the development of ER-negative tumors.

Suppression of tumor development by LGD1069 is relatively modest in comparison to the dramatic suppression reported by Gottardis et al. (23) in the NMU-induced rat mammary tumor model. In the NMU rat model, a 90% reduction in tumor burden and incidence was observed (23). As discussed previously (17), the differences in efficacy may be because of mutations that arise from different pathways in these two models i.e., the NMU carcinogen induces ras mutations, whereas the SV40 Tag inactivates the p53 and Rb tumor suppressor gene proteins. Just as we observed in the present study, Gottardis et al. (23) also observed no signs of toxicities in the rats treated with LGD1069.

Plasma levels of TTNPB were undetectable by the methods used. Submicromolar concentrations of TTNPB were beyond the sensitivity of our high-performance liquid chromatography assay. However, concentrations were high enough to induce significant toxicities in the mice. Fig. 6A shows the average plasma concentration over time after a single dose of LGD1069 (either 10 or 100 mg/kg). As shown in Table 3, the T_{max} was 30 minutes after a single dose of LGD1069 for both doses. Peak plasma concentrations achieved after a single dose were 2.61 and 58.01 μM for 10 mg/kg and 100 mg/kg, respectively.

Fig. 6B shows the average plasma concentration over time after a dose of LGD 1069 in mice that were chronically treated with either 10 mg/kg (low dose) or 100 mg/kg (high dose) of LGD1069 for 28 days. As shown in Table 3, the T_{max} after chronic dosing was 30 min for the high dose and 60 min for the low dose. Plasma concentrations of LGD1069 after a single dose in chronically treated mice reached a lower maximal level (3.72 and 8.16 μM for 10 and 100 mg/kg, respectively) than compared with those in the single dose. Area under the curve values at 28 days were 43% and 87% lower than the area under the curve values after a single dose at the 10 and 100 mg/kg doses, respectively. These results suggest that chronic dosing of LGD1069 induces enzymes, which metabolize LGD1069.

The results demonstrate that doses of 100 mg/kg used in these studies can achieve peak plasma levels up to 8 μM in chronically treated animals. These levels are consistent with levels that inhibit breast cell growth in vitro.
recently, studies by Bischoff et al. (28) have shown that a combination of LGD1069 with the antiestrogen tamoxifen had an increased efficacy on NMU-induced mammary tumors.

On the basis of preclinical studies of LGD1069, this RXR-selective retinoid has already been tested in human clinical trials. In Phase I clinical trials for the treatment of cancer (29, 30) LGD1069 was found to suppress the growth of cutaneous lymphoma and has now been approved for the treatment of cutaneous T-cell lymphoma (31). The first cancer prevention trial using LGD1069 has just been activated at our institution.

Acknowledgments

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References


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