The Retinoid X Receptor-Selective Retinoid, LGD1069, Down-regulates Cyclooxygenase-2 Expression in Human Breast Cells through Transcription Factor Crosstalk: Implications for Molecular-Based Chemoprevention

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Abstract

Retinoids and their derivatives can suppress the development of cancer in animals and in humans. We and others have shown that retinoid X receptor (RXR)-selective retinoids or "rexinoids" suppress the development of breast cancer in several animal models with minimal toxicity. LGD1069 (Bexarotene) is a potent RXR-selective retinoid with reduced toxicity compared with naturally occurring retinoids. In this study, we investigated the expression of LGD1069-modulated biomarkers. We previously did cDNA array analysis of LGD1069-treated breast cells using Affymetrix microarrays. These studies identified many LGD1069-regulated genes, one of which was cyclooxygenase-2 (COX-2). Because COX-2 inhibitors have been shown to prevent cancer in other model systems, we investigated whether LGD1069 inhibits the expression of COX-2 in mammary tissue and in normal human mammary epithelial cells (HMEC). In mouse mammary tumor virus-erbB2 mice treated with LGD1069, there was a marked decrease of COX-2 expression in both normal and malignant mammary tissues. The effect of LGD1069 on COX-2 expression was also investigated in normal human breast cells. COX-2 expression was markedly reduced by treatment with LGD1069 at the RNA and protein level in normal HMECs; LGD1069 suppressed COX-2 promoter activity. We also showed that LGD1069 inhibited activator protein (AP-1)-dependent transcription in these breast cells, and that suppression of COX-2 expression was due to sequestration of CBP/p300. These results from *in vivo* and *in vitro* studies suggest that LGD1069, an RXR-selective retinoid, inhibits COX-2 expression by suppression of COX-2 transcription in part through transrepression of the AP-1 transcription factor. Thus, RXR-selective retinoids that inhibit AP-1 activity and suppress COX-2 expression may be particularly promising drugs for breast cancer prevention. Furthermore, such RXR-selective retinoids may be most useful in combination with antiestrogens for more effective prevention of breast cancer in women at high risk of this disease. (Cancer Res 2005; 65(8): 3462-9)

Introduction

Breast cancer is the second leading cause of cancer death in women in the United States. Little progress has been made over the last 20 years in the treatment of metastatic breast cancer. Therefore, there has been increased emphasis on detecting breast cancer early or preventing its development. Recently, preventive therapy using selective estrogen receptor modulators has been shown to be a promising strategy for reducing the risk of breast cancer. Several large clinical trials have shown that the antiestrogens, tamoxifen and raloxifene, reduce the risk of breast cancer by 50% or more (1–3). However, whereas these agents greatly reduce the incidence of estrogen receptor–positive breast cancer, they do not reduce the incidence of estrogen receptor–negative breast cancer (1–3). Thus, additional strategies for the prevention of estrogen receptor–negative breast cancer are urgently needed.

Retinoids are biologically active derivatives of vitamin A that regulate the growth and differentiation of normal and malignant cells (4). Retinoids inhibit the growth of several human cancer cell lines and suppress tumor formation in animals (5–7). However, the use of these agents is limited by their toxicity, which can include chelitis, hypertriglyceridemia, and hepatosplenomegaly (8).

Retinoids act by binding to the retinoic acid receptor (RAR) and retinoid X receptor (RXR) nuclear retinoid receptors (each with three subtypes, α, β, and γ). RAR and RXR proteins act as ligand-dependent transcription factors, which can modulate the transcriptional activity of retinoid receptor target genes by binding as RAR/RXR heterodimeric complexes to specific RAR or RXR response elements within gene promoters (9, 10). In addition, RXR protein also dimerizes with other nuclear hormone receptors such as vitamin D receptor, thyroid hormone receptors, PPAR-α, and -γ and orphan receptors (reviewed in refs. 11–14). 9-*cis*-Retinoic acid (Alitretinoin) binds both RAR and RXR receptors and has been shown by others and us to suppress the development of breast cancer in several animal models (7, 15, 16). However, 9-*cis*-retinoic acid is quite toxic in mice and humans (8). LGD1069 (Bexarotene) is an RXR-selective retinoid that does not activate RAR-dependent genes and thus is less toxic than naturally occurring retinoids or RAR-selective retinoids. Previous studies have shown that LGD1069 effectively prevents the development of estrogen receptor–positive mammary tumors in nitrosomethylurea-treated rats (17, 18). In addition, we have previously shown that LGD1069 suppresses the development of estrogen receptor–negative mammary tumors in transgenic mice (19, 20).

Note: G. Kong and H-T. Kim contributed equally to this work.

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We are currently investigating the mechanism by which LGD1069 suppresses mammary tumorigenesis. As part of these studies, we have identified retinoid-modulated genes using Affymetrix microarrays. These studies have shown that cyclooxygenase-2 (COX-2) is down-regulated by LGD1069. In this study, we have confirmed the down-regulation of COX-2 by LGD1069 in normal and cancerous mammary gland tissues from mouse mammary tumor virus (MMTV)-erbB2 transgenic mice and in estrogen receptor–negative normal human mammary epithelial cells (HMEC) *in vitro*. Further investigation showed that LGD1069 inhibits activator protein (AP-1)–mediated activation of COX-2 transcription by limiting the amount of available CBP/p300 integrator protein (transcriptional squelching). These data suggest that LGD1069, an RXR-selective retinoid, suppresses tumorigenesis in part through transrepression of the AP-1 transcription factor leading to decreased expression of COX-2.

Materials and Methods

Retinoids. The RXR-selective retinoid used in this study, LGD1069 (Bexarotene, Targretin), was obtained from Ligand Pharmaceuticals, Inc. (San Diego, CA). It was received as a lyophilized powder and suspended in DMSO. This solution was added to culture medium to achieve a final concentration of 1 μmol/L LGD1069 and 0.1% DMSO. The vehicle (DMSO) was used as a control in all experiments at a final concentration of 0.1%.

***In situ* hybridization for cyclooxygenase-2.** Levels of COX-2 mRNA in MMTV-erbB2 transgenic mice of both groups treated with LGD1069 (10 and 100 mg/kg) and vehicle were measured by using a method of nonradioactive *in situ* hybridization as described previously (21). Briefly, the tissue sections first underwent treatment with 0.2 N HCl and proteinase K, respectively, after deparaffinization and rehydration. The slides were then postfixed with 4% paraformaldehyde and acetylated in freshly prepared 0.25% acetic anhydride in a 0.1 mol/L triethanolamine buffer. The slides were then prehybridized at 42°C with a hybridization solution containing 50% deionized formamide, 2× SSC, 2× Denhardt's solution, 10% dextran sulfate, 400 μg/mL yeast tRNA, 250 μg/mL salmon-sperm DNA, and 20 mmol/L DTT in diethylpyrocarbonate-treated water. Next the slides were incubated in 50 μL per slide hybridization solution containing 20 ng of a freshly denatured digoxigenin-cRNA probe at 42°C for 4 hours. After that, the slides were washed for 2 hours in 2× SSC containing 2% normal sheep serum and 0.05% Triton X-100, and then for 20 minutes at 42°C in 0.1× SSC. For color reaction, the slides were incubated for 30 minutes at 23°C in 0.1 mol/L maleic acid and 0.15 mol/L NaCl (pH 7.5, buffer 1) containing 2% normal sheep serum and 0.3% Triton X-100 and then incubated overnight at 4°C with a sheep anti-digoxigenin antibody. After being washed in buffer twice, the color was developed in a chromogen solution for 4 hours. The slides were then mounted with a cover glass in Aqua mounting medium (Fisher, Houston, TX). The stained sections were reviewed and scored independently by two pathologists (G. Kong and X.C. Xu) with an Olympus microscope. The sections were classified as positive or negative staining; 10% or more positive epithelial cells as strongly positive, 0% to 10% as weakly positive.

Quantitative reverse transcription-PCR. Total RNA was isolated using the Qiagen RNeasy kit (Qiagen, Inc., Valencia, CA). RNA samples were treated with DNase I and then cDNA was made from each sample. cDNAs of the COX-2 gene and an internal reference gene (β-actin) were quantified using a fluorescence-based real-time detection method (ABI PRISM 7700 Sequence Detection System, TaqMan; Perkin-Elmer Applied Biosystems, Foster City, CA) in the ABI manual. The PCR reaction mixture consisted of 300 nmol/L each of the primers, 100 nmol/L probe, 0.025 units/μL of Taq polymerase, 125 μmol/L each of deoxynucleotide triphosphate, 3 mmol/L $MgCl_2$, and 1× Taq polymerase buffer. Cycling conditions were 94°C for 1 minute, followed by 40 cycles at 94°C for 12 seconds, and 60°C for 1 minute. The following is the sequence for the probe, primers, and standard:

COX-2: probe: 5′-CCA CAA TCT GGC TGA GGG AAC ACA AC
forward primer: 5′-TCT GGT GCC TGG TCT GAT G
reverse primer: 5′-CCC ATT CAG GAT GCT CCT G
β-actin: probe: 5′-ATC AAG ATC ATT GCT CCT CCT GAG CGC
forward primer: 5′-CCC TGG CAC CCA GCA C
reverse primer: 5′-GCC GAT CCA CAC GGA GTA C

Standard curves for the quantification of COX-2 and β-actin were constructed using each standard synthetic template and genome equivalent copies were calculated from the standard curve. For each sample, TaqMan PCR reactions were done in triplicate for each gene of interest and reference gene to normalize for input cDNA. The ratio between the values obtained provided relative gene expression levels.

Western blotting analysis. Cell lysates were prepared by treating cells with lysis buffer [50 mmol/L Tris-HCl (pH 8.0), 2% SDS, and protein kinase inhibitor cocktail]. Lysates were sheared using a 22-gauge needle and centrifuged at 10,000 × *g* for 30 minutes. The proteins were resolved on a 10% SDS-polyacrylamide gel, and then were transferred to a nitrocellulose membrane, and the membrane was then blocked in 5% nonfat dry milk TBST [10 mmol/L Tris-HCl (pH 8.0), 150 mmol/L NaCl, 0.05% Tween 20] at room temperature for 1 hour. Primary antibody was diluted at 1:500 in 1% nonfat dry milk TBST for antibodies against COX-2 (Cayman Chemical, Ann Arbor, MI), and for anti-p300 antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). The membrane was incubated at room temperature for 2 to 3 hours and washed thrice with TBST for 10 minutes. The membrane was then incubated with corresponding horseradish peroxidase-conjugated secondary antibodies in 1% nonfat dry milk/TBST at room temperature for 1 hour and washed thrice with TBST for 10 minutes. The blots were probed with the enhanced chemiluminescence Western blot detection system (Amersham, United Kingdom) according to the manufacturer's instructions.

Enzyme immunoassay for prostaglandin E_2 synthesis. HMEC were plated in six-well plates and then treated with vehicle (DMSO) or 1 μmol/L LGD1069 for 24 hours. The medium was then replaced with completed medium to which 10 μmol/L sodium arachidonate was added. After 30 minutes, the medium was collected to determine prostaglandin E_2 (PGE_2) synthesis by enzyme immunoassay according to the manufacturer's instruction (Cayman Chemical), and normalized to protein concentrations.

Cell culture and cell proliferation assay. Normal HMEC were obtained from Clonetics (San Diego, CA). They were obtained as primary cultures derived from healthy women who had undergone reduction mammoplasties. Cells between passages 6 and 12 were used. Cells were grown and maintained in mammary epithelial basal medium supplemented with 13 mg/mL bovine pituitary extract, 0.5% serum, 5 μg/mL insulin, 10 ng/mL human recombinant epidermal growth factor, 0.5 μg/mL hydrocortisone, 50 μg/mL gentamicin, and 50 μg/mL amphotericin-β (Clonetics). Cells were maintained in a humidified environment at 37°C with 5% CO_2 in air. Cell growth was determined using the Cell Titer 96 Aqueous Non-Radioactive Cell Proliferation Assay (Promega, Madison, WI) according to the protocol provided by the manufacturer.

Plasmids and transfections. The COX-2 promoter constructs (−327/+59, −327/+59 with a mutated CRE) have been previously described (22, 23). Col-Z-Luc reporter for AP-1 activity was a generous gift from Dr. J. Kurie (M.D. Anderson Cancer Center, Houston, TX). Tk-luc was obtained from Promega. Co-activator p300 expression vector was a gift from Dr. Suzanne Fuqua (Baylor College of Medicine, Houston, TX). For transient transfection, 2×10^5 cells per well were plated in six-well chambers. After 24 hours, the cells were transfected with 1 μg of promoter construct and 0.5 μg of tk-luc DNAs using Fugene 6 reagent (Roche Diagnostics Corp., Indianapolis, IN) according to the manufacturer's instructions. After 24 hours of incubation, cells were treated with 1 μmol/L of LGD1069 for 24 hours. Luciferase activity was measured in the cellular extract as previously described (24).

Electrophoretic mobility shift assay. To assess transcription factor DNA binding activity, cells were cultured for 2 to 4 days, then harvested, and nuclear extracts were prepared as previously described (24). For binding studies, double-stranded oligonucleotides containing AP-1 or CRE consensus elements were used. Double-stranded oligonucleotides were phosphorylated at the 5′-end with [γ-^{32}P]ATP and T4 polynucleotide

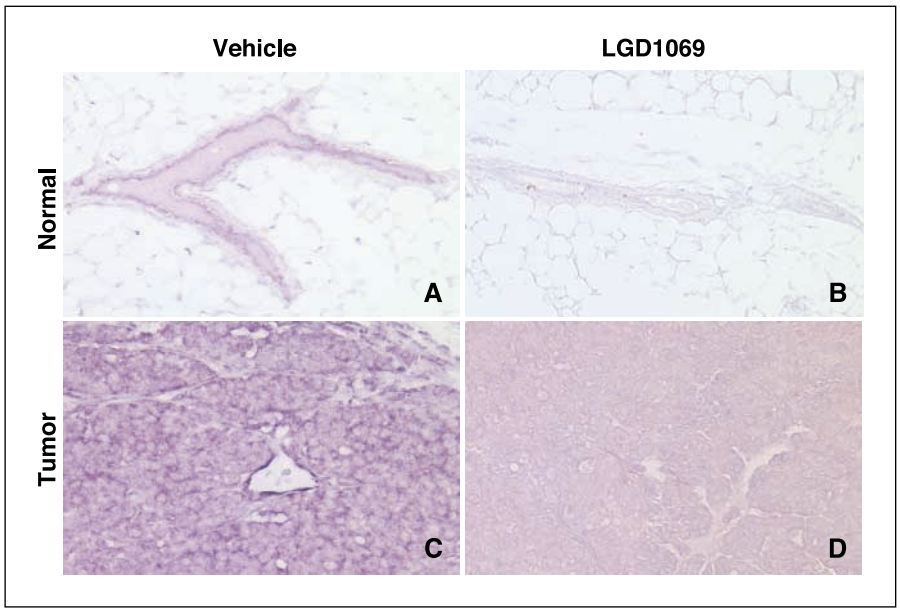

Figure 1. *In situ* hybridization analysis of COX-2 expressions in MMTV-erbB2 mice treated with LGD1069. *In situ* hybridization of COX-2 expression was done in MMTV-erbB2 transgenic mice treated with vehicle (control) and LGD1069 (10 and 100 mg/kg). *A* and *C,* normal ductal epithelium and carcinoma in control group, respectively; *B* and *D,* normal duct and carcinomas in treated group with 100 mg/kg, respectively.

kinase. The binding reaction was then done by incubating 5 μg of nuclear protein from the cell lysates in 20 mmol/L HEPES (pH 7.9), 10% glycerol, 300 μg of bovine serum albumin, and 1 μg of poly(dI·dC) in a final volume of 10 μL for 10 minutes at 25°C. The labeled oligonucleotide was added to the reaction mixture and allowed to incubate for an additional 20 minutes at 25°C. The samples were electrophoresed on a 4% nondenaturing polyacrylamide gel. The gel was then dried and subjected to autoradiography at −80°C.

Chromatin immunoprecipitation assay. HMEC cells were exposed to vehicle or 1 μmol/L LGD1069 for 4 hours and were treated with 1% formaldehyde for 10 minutes to cross-link histones to DNA before being sonicated. Specific antibodies for c-Jun (Calbiochem), and p300 (Santa Cruz Biotechnology) were used for immunoprecipitation of protein-DNA complexes. After cross-link reversal and DNA purification, primers flanking the AP-1 site of the MMP1 promoter (−242/−3) or a region that contains the COX-2 CRE site (−139/+36) were used in PCR amplification. PCR primers produced 239 bp DNA fragments for AP-1/MMP1, and primers for the COX-2 CRE site amplified 175 bp DNA fragments. Amplified DNA was fractionated on an agarose gel and visualized after ethidium bromide staining.

Data analysis and statistics. The effect of treatment with the retinoid LGD1069 was evaluated in a number of assays. The resulting data were summarized with means and SE. For both reverse transcription-PCR and luciferase data, tests for equality of variances among groups (tested by F_{max} test) were significant, indicating that the assumption of equal variances was violated. Data were therefore transformed by taking logarithms to stabilize variances. Log-transformed reverse transcription-PCR data were compared using two sample *t* tests. For luciferase assays, log-transformed data from two or more experiments were combined and analyzed using two-way ANOVA to evaluate the effect of LGD1069 after accounting for experiment to experiment differences in level of luciferase activity. The *in vivo* association between COX-2 expression and LGD1069 treatment in normal and tumor tissue was assessed by contingency table analysis. We used the Fisher-Freeman-Halton test, which is a generalization of Fisher's exact test for greater than 2 × 2 tables, to test globally for any difference, followed by Fisher's exact test comparing vehicle to individual doses to determine which groups differed.

Table 1. LGD1069 down-regulates COX-2 expression in MMTV-c-erbB2 transgenic mice

	COX-2 expression			
	Normal glands		Tumors	
	Negative	Positive	Negative	Positive
Vehicle (n = 9)	0	9	1	8
LGD1069, 10 mg/kg (n = 3)	2	1	1	2
LGD1069, 100 mg/kg (n = 4)*	3	1	3	0

*n = 3 for tumors.

Results

We have previously done Affymetrix microarray analysis in an attempt to identify the LGD1069-regulated genes.[5] These studies showed that COX-2 was consistently down-regulated after 24 hours of LGD1069 treatment. Therefore, we investigated the effect of LGD1069 on COX-2 expression in mammary cells in MMTV-erbB2 mice and in normal HMECs.

LGD1069 down-regulates cyclooxygenase-2 expression *in vivo* in normal and malignant cells. We first investigated whether LGD1069 treatment caused suppression of COX-2 expression *in vivo*. Previously, we treated MMTV-erbB2 transgenic mice with LGD1069 in an attempt to suppress tumor formation. These studies showed that LGD1069 significantly reduced the incidence of mammary tumors in these mice, and that LGD1069 also prolonged the time to tumor development in the mice that did develop tumors (20). In the current study, we used mammary gland tissues from these animals to measure COX-2 RNA expression in vehicle- and LGD1069-treated mice using *in situ* hybridization. Normal ductal epithelium and breast tumor tissues from MMTV-erbB2 transgenic mice of the vehicle group showed constitutively high expression of COX-2, whereas the normal and malignant breast tissue from mice treated with LGD1069 had reduced expression of COX-2 (Fig. 1). These data are summarized in Table 1; the mammary tissue from

[5] Unpublished results.

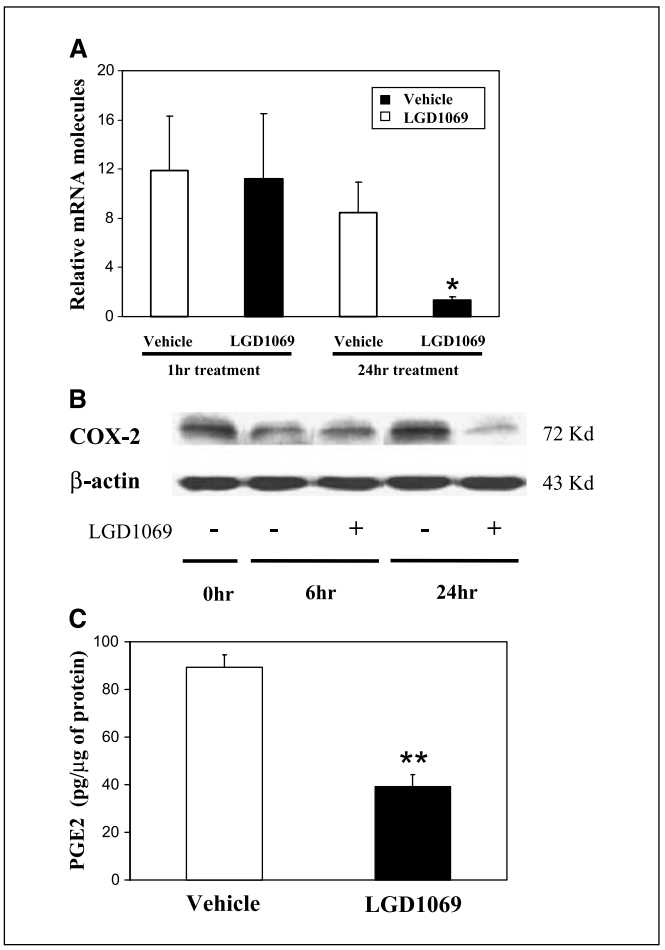

Figure 2. LGD1069 down-regulates COX-2 mRNA and protein expression, and suppresses PGE_2 synthesis in HMECs. HMECs were treated with vehicle (DMSO) and 1 μmol/L LGD1069. Real-time quantitative reverse transcription-PCR were done on total RNA isolated from HMECs treated for 1 and 24 hours. Results shown here are the average with the SE of normalized mRNA expression levels with β-actin (*A*). LGD1069 treatment significantly reduces COX-2 expression at 24 hours (*P = 0.048). Cell lysates of each sample treated for 6 and 24 hours were used for Western blot analysis for COX-2 expression (*B*). Cells were treated with LGD1069 for 24 hours before PGE_2 synthesis was measured by enzyme immunoassay (*C*). LGD1069 significantly suppresses PGE_2 synthesis (**P = 0.002). *Columns,* mean; *bars,* SE.

mice treated with vehicle showed expression of COX-2 in both normal (nine of nine cases) and tumor tissues (eight of nine cases), whereas the mammary tissue from mice treated with high dose of LGD1069 (100 mg/kg) had reduced expression of COX-2 in normal ductal epithelium (three of four cases had no expression) and tumor tissues (three of three cases had no expression). Fisher-Freeman-Halton exact tests for independence indicate that in both normal glands and tumors, COX-2 positivity is dependent on treatment (P = 0.007 and P = 0.02, respectively). Fisher's exact tests to identify which treatments differ from control indicate that in normal glands, the expression of COX-2 in glands from low dose (10 mg/kg) and high dose (100 mg/kg) treated mice is significantly lower than that in glands from control mice (P = 0.05 and P = 0.014, respectively). In tumors, COX-2 expression in only high dose–treated mice differs from control (P = 0.018). These data indicate that LGD1069 suppresses COX-2 expression in mammary cells *in vivo*.

LGD1069 down-regulates cyclooxygenase-2 expression *in vitro* in normal human mammary epithelial cells and inhibits cell growth. We next investigated the effect of LGD1069 on human breast cells. For these experiments, we used normal HMEC, and to measure the changes of the expression of COX-2 RNA, we performed quantitative real-time reverse transcriptase-PCR. As shown in Fig. 2*A*, treatment with LGD1069 decreased levels of COX-2 mRNA in HMECs by 80% after 24 hours. We next measured the effect of LGD1069 treatment on COX-2 protein expression, and PGE_2 synthesis. Western blot analysis showed that treatment with 1 μmol/L of LDG1069 led to suppression of COX-2 expression with >80% reduction after 24 hours (Fig. 2*B*), and >50% suppression of PGE_2 synthesis was detected by using enzyme immunoassay (Fig. 2*C*). We also examined the effect of LGD1069 on cell growth of normal HMECs. LGD1069 (at 1 μmol/L) inhibits the growth of these cells as shown in Fig. 3. We also observed that specific COX-2 inhibitors suppress the growth of normal human breast epithelial cells (data not shown).

LGD1069 inhibits cyclooxygenase-2 promoter activity. We next investigated the effect of LGD1069 on the activity of the COX-2 promoter. Transient transfections were done using a human COX-2 promoter luciferase construct (−327/+59). Treatment with LGD1069 for 24 hours resulted in a decrease in COX-2 promoter activity. Figure 4 shows the results of these experiments and shows a 41% reduction in COX-2 promoter activity after LGD1069 treatment. This reduction suggests that LGD1069 suppresses COX-2 transcription through this region of the promoter. The fact that LGD1069 caused a greater decrease in levels of COX-2 mRNA (Fig. 2) than reduction in COX-2 promoter activity suggests that LGD1069 may also affect RNA expression through additional mechanisms. Transient transfection was also done with −327/+59 COX-2 promoter construct containing a mutated CRE site. Mutation of the CRE site abolished promoter activity, indicating that the site is required for COX-2 expression in HMEC (Fig. 4*B*). In addition, we measured the effect of other retinoids on COX-2 promoter activity using the human COX-2 promoter luciferase −327/+59 construct. Figure 4*C* shows that naturally occurring retinoids (9-*cis*-RA, and all-*trans*-RA) also suppress COX-2 transcriptional activation. Thus, the down-regulation of COX-2 expression occurs with retinoids that bind RAR as well as those that bind RXR.

LGD1069 inhibits cyclooxygenase-2 transcriptional activation by suppressing AP-1 activity. To define the molecular mechanism by which LGD1069 inhibits COX-2 expression, we examined the effect of LGD1069 on transcription factor activity.

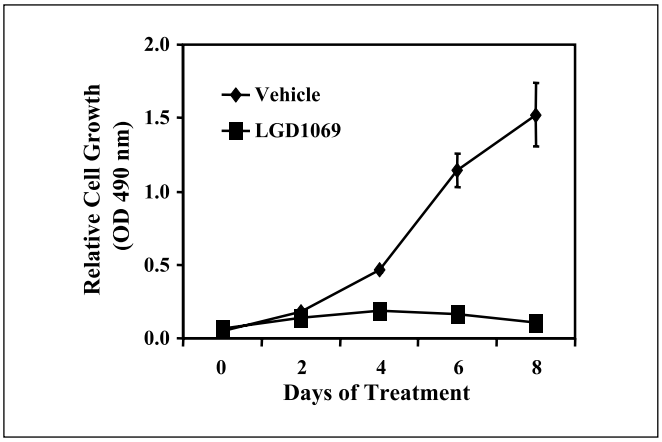

Figure 3. LGD1069 inhibits HMEC growth *in vitro*. Cells were treated with 1 μmol/L LGD1069 and vehicle (DMSO) and proliferation was measured using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. *Points,* mean; *bars,* ± SE.

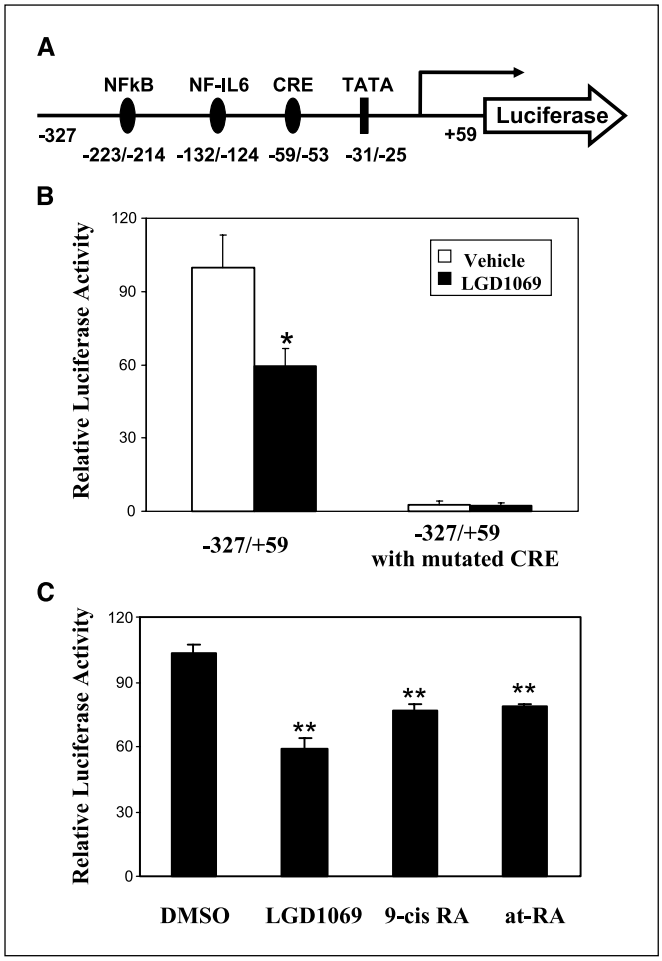

Figure 4. LGD1069 inhibits COX-2 promoter activity. *A,* schematic of human COX-2 promoter; *B,* COX-2 promoter activity: HMECs were transfected with 0.8 μg of COX-2 promoter constructs (−327/+59, and −327/+59 with a mutated CRE site) and 0.2 μg of tk-luc construct as a reference. LGD1069 treatment reduced luciferase reporter activity of the COX-2 promoter by 41% (*$P = 0.052$); *C,* COX-2 promoter activities were measured after treatment with 1 μmol/L LGD1069, 9-*cis*-RA, and all-*trans*-RA each for 24 hours. All retinoid effects were statistically significant (** all $P < 0.021$). Reporter activities are measured in cell extracts. Luciferase activity has been normalized with tk-luc activity. *Columns,* mean; *bars,* SE.

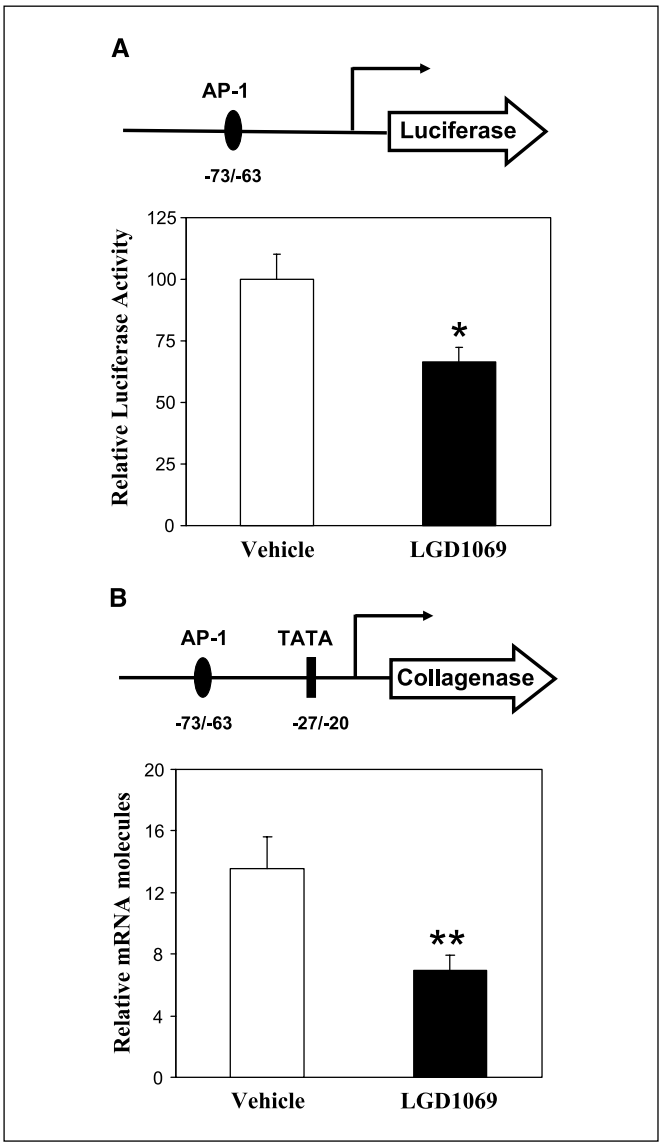

Figure 5. Effect of LGD1069 on AP-1 transcriptional activity and AP-1-dependent collagenase gene transcription. *A,* AP-1 transcriptional activity was measured using the Col-z-luc construct. Luciferase activity was normalized with tk-luc activity. LGD1069 treatment significantly reduced AP-1 activity (*$P = 0.047$); *B,* quantitative reverse transcription-PCR for collagenase expression was done to investigate the effect of LGD1069 on an AP-1-dependent gene. LGD1069 treatment for 24 hours led to an ~49% reduction in collagenase RNA expression (**$P = 0.046$). *Columns,* mean; *bars,* SE.

Previous studies have shown that the AP-1 transcription factor stimulates the expression of the COX-2 gene (25). Therefore, we investigated whether LGD1069 affects AP-1 transcriptional activity, AP-1-dependent DNA binding activity, and the expression of AP-1-dependent downstream genes. As shown in Fig. 5*A*, LGD1069 inhibits the activity of the Col-Z-luc AP-1 reporter construct (the activity of the Col-Z-luc reporter is reduced by 34%). Consistent with this finding, LGD1069 suppressed the expression of collagenase (MMP-1) an AP-1-dependent gene by 49% (Fig. 5*B*). We also determined whether LGD1069 affects the expression level of AP-1 proteins, c-Jun, c-Fos, and cAMP-responsive element binding protein (CREB). Levels of c-Jun, c-Fos, and CREB were unaffected by treatment with LGD1069 (data not shown). We next measured the effect of LGD1069 treatment on AP-1 or CRE-dependent DNA binding activity using double-stranded oligonucleotides containing an AP-1 consensus element or a CRE consensus element. As shown in Fig. 6, treatment of HMECs with LGD1069 did not change AP-1-dependent DNA binding activity and CRE-dependent DNA binding activity.

The fact that the rexinoid does not affect AP-1 DNA-binding raises the possibility that the rexinoid suppresses AP-1-dependent transcription through squelching of co-activators. Thus, we further investigated the role of p300, a co-activator of AP-1-mediated transcription. We first examined the effect of retinoid treatment on COX-2. As shown in Fig. 7*A*, we observed that COX-2 protein expression was suppressed by LGD1069, 9-*cis*-RA, or all-*trans*-RA after 24-hour treatment but not after 6-hour treatment. All these retinoids reduced COX-2 protein by >50% of the control level. We next examined the effect of these retinoids on the expression of the co-activator p300. There was no reduction in p300 expression after retinoid treatment at 6 or 24 hours (Fig. 7*A*). In an attempt to overcome this retinoid-induced suppression of COX-2 promoter activity, we overexpressed increasing amounts of p300 in HMEC

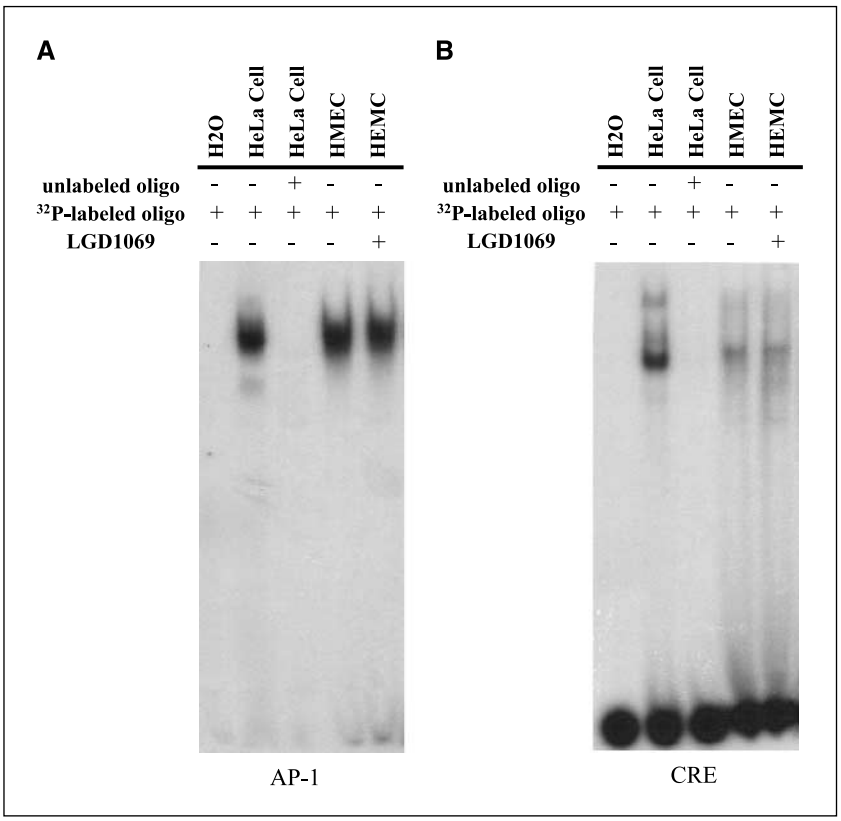

Figure 6. LGD1069 does not inhibit AP-1 and CRE DNA binding activities. AP-1 and CRE binding activity was measured by gel shift. Nuclear protein (5 μg) from HMEC treated with 1 μmol/L LGD1069 and vehicle was incubated with ^{32}P-labeled double-stranded oligonucleotide containing the AP-1 consensus elements (*A*), or CRE of COX-2 promoter (*B*). Nuclear protein (5 μg) from HeLa cells was used for control.

cells treated with LGD1069 and transfected with the COX-2-luc reporter construct. When the co-activator p300 was overexpressed in the HMEC cells, the suppressive effect of LGD1069 on COX-2 promoter activity was reversed in a dose-dependent manner (Fig. 7*B*).

Next, chromatin immunoprecipitation assays were done using IgG or antibodies to c-Jun or p300. Figure 8 shows results of these studies. Antibodies to c-Jun immunoprecipitated both AP-1 sequences from MMP-1 promoter (−242/−3) and CRE sequences from the COX-2 promoter (−139/+36). No difference was observed in the recruitment of c-Jun between vehicle- and LGD1069-treated cells. However, immunoprecipitation of p300 that was present in complexes that bound these AP-1 or CRE sites was significantly reduced by LGD1069 treatment. These data suggest that LGD1069 inhibits COX-2 transcription not by inhibiting the binding of AP-1 or CREB to the CRE site of the COX-2 promoter, but instead by reducing the amount of p300/CBP recruited to the promoter. Thus, RXR-selective retinoids (rexinoids) suppress COX-2 expression by sequestering p300/CBP and making it unavailable for co-activation of AP-1 at the COX-2 promoter, and thereby suppressing the AP-1-mediated transcription of COX-2.

Discussion

Retinoids have been shown to be promising agents for the prevention and treatment of several human cancers including leukemia, head and neck cancer, skin cancer, and possibly breast cancer (26–28). We have previously shown that 9-*cis*-RA and the RXR-selective retinoid, LGD1069 suppress mammary tumor development in transgenic mice (7, 19, 20). In this report, we show that the RXR-selective retinoid LGD1069 inhibits the growth of normal human breast epithelial cells, and that LGD1069 suppresses the expression of COX-2 in normal HMECs *in vitro*, and in normal and malignant breast cells in MMTV-erbB2 transgenic mice. It is therefore likely that the RXR-selective retinoid suppresses the growth of normal and premalignant mammary epithelial cells, and thus prevents them from acquiring additional genetic mutations which could induce cancer.

Our results suggest that LGD1069 inhibits transactivation of the COX-2 promoter and that the CRE within this promoter is required for expression. Previous studies have shown that ligands of nuclear receptors, including retinoids, suppress COX-2 transcription by antagonizing AP-1 activity (29). We therefore investigated the effect of LGD1069 on AP-1 activity in breast cells. These studies show that LGD1069 treatment suppresses the expression of the AP-1-regulated gene (collagenase), does not affect the binding of nuclear proteins to DNA containing either AP-1 or CRE consensus elements, and sequesters p300/CBP from the COX-2 promoter. Thus, these results show that RXR-selective ligands inhibit COX-2 expression, at least in part, by inhibiting AP-1-dependent transcription of the COX-2 gene through squelching of essential co-activators. It is notable that the suppression of COX-2 expression observed was greater than the reduction of AP-1 or CRE transactivation activity. These results suggest that LGD1069 may also affect COX-2 expression through other regulatory pathways or mechanisms, such as through transrepression of other transcription factors or through modulation of RNA stability.

Several studies of COX-2 expression in transgenic mice and human breast tumors suggest that overexpression of COX-2 is also involved in the development of breast cancer. COX-2 expression was detected in 45% to 70% of human primary breast cancers, whereas it is usually not detected in normal ductal epithelium (30–33).

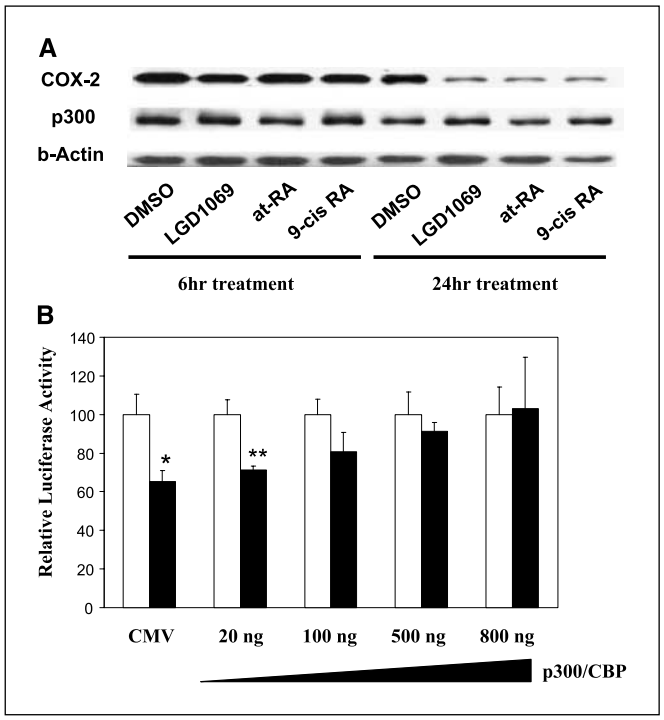

Figure 7. Overexpression of p300 reverses LGD1069-mediated suppression of COX-2 promoter activity. *A,* Western blot analysis was done for COX-2 and p300 expression after treatment with different retinoids; *B,* COX-2 promoter activity was measured after different amounts of p300 expression vector were transfected. Luciferase reporter activities were measured in cell extracts and normalized with tk-luc activity. LGD1069 treatment significantly reduced luciferase reporter activity in cytomegalovirus-vector control cells (*$P = 0.041$), and the cells transfected with low amount (20 ng) of p300 expression vector (**$P = 0.022$). Overexpressing p300 reversed the suppressive effect of LGD1069 on COX-2 promoter activity in a dose-dependent manner. *Columns,* mean; *bars,* SE. □, vehicle; ■, LGD1069.

In addition, Liu et al. (34) showed that forced expression of COX-2 was sufficient to induce mammary tumors in multiparous MMTV-COX-2 transgenic mice. Interestingly, in human breast cancer, high expression of COX-2 is significantly associated with an estrogen receptor–negative phenotype, high expression of erbB2, and poor prognosis (31, 33). Recent studies by Howe et al. (35) also show that the COX-2 inhibitor, celecoxib suppresses the development of mammary cancer in MMTV-erbB2 transgenic mice. The present results showing that LGD1069 can suppress the expression of COX-2, along with previous studies showing that LGD1069 suppresses the development of estrogen receptor–negative tumors in animals, suggest that LGD1069 would be particularly effective at suppressing the development of the estrogen receptor–negative human breast tumors that otherwise would have a poor prognosis.

Prostaglandins, the products of COX-2 enzyme activity, affect cell proliferation, tumor growth, angiogenesis, and immune response in normal and malignant cells (36). COX-2 derived prostaglandins might act on malignant epithelial cells or on the surrounding stroma to promote tumor development. Although the mechanistic basis underlying this phenomenon is incompletely understood, recent experiments have identified the key prostaglandin signaling pathways responsible for the tumorigenesic effect of COX-2 (36, 37). COX-2 inhibitors inhibit cancer cell growth and suppress tumor formation in animal models (reviewed in ref. 36). Clinical studies have shown that a selective COX-2 inhibitor caused the regression of colorectal polyps in individuals with familial adenomatous polyposis (38).

We have previously shown that LGD1069 suppresses the development of mammary tumors in MMTV-erbB2 transgenic mice (20). We now show that LGD1069 suppresses growth of HMECs and also inhibits COX-2 expression *in vitro* in HMECs and *in vivo* in mammary tissue derived from MMTV-erbB2 transgenic mice. Thus, these results indicate that LGD1069 may suppress the formation of breast cancer, in part, by reducing COX-2 expression.

The effects of retinoids are mainly mediated by RARs and RXRs. Both receptor types are encoded by three distinct genes (α, β, and γ), which bind to their retinoic acid response element-containing target genes and regulate transcription (11, 12). In addition, retinoids also affect other transcription factors through transcriptional factor crosstalk (39). We and others have shown that retinoids have anti-AP-1 activity and that they can inhibit the expression of genes that do not contain retinoic acid response

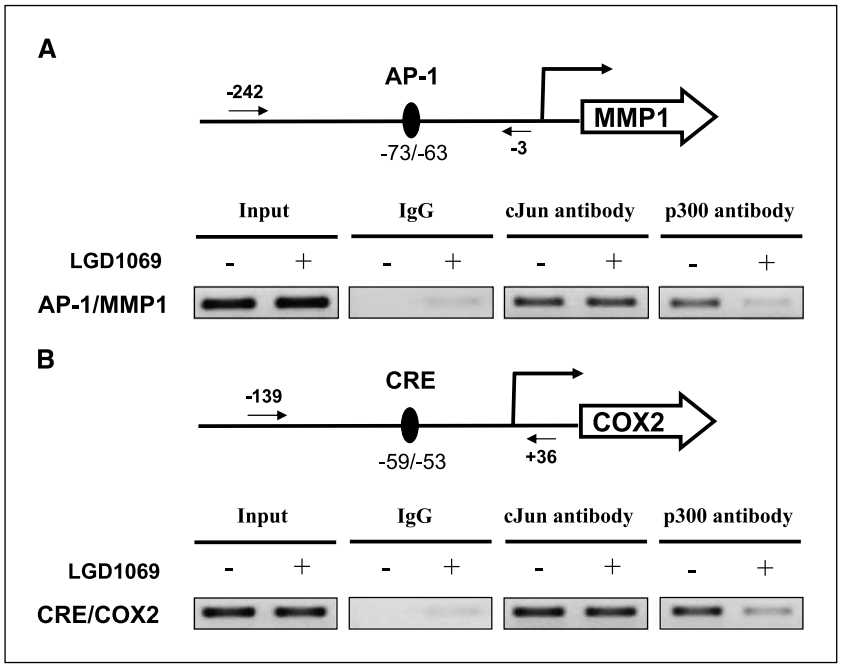

Figure 8. LGD1069 decreases p300 co-activator at MMP-1 and COX-2 promoters. Chromatin immunoprecipitation assays are shown. HMECs had been exposed to vehicle (DMSO) or 1 μmol/L LGD1069 for 4 hours, then cross-linked protein-DNA complexes were immunoprecipitated with IgG, c-Jun, and p300–specific antibodies. Primers (*A*), flanking the AP-1 site of MMP1 promoter (−242/−3) or (*B*), a region of the COX-2 promoter that contains a CRE site (−139/+36) were used in PCR amplification. Antibodies to c-Jun immunoprecipitated both AP-1 and CRE sequences from the MMP-1 and COX-2 promoters. Immunoprecipitation of p300 associated with DNA containing either AP-1 or CRE sites was significantly reduced by LGD1069 treatment. Genomic DNA was used as a positive control (*Input*).

elements within their promoters (12, 25, 27, 40). Naturally occurring retinoids, such as all-*trans*-RA (tretinoin), 9-*cis*-RA, 13-*cis*-RA, and retinyl acetate have been shown to suppress phorbol myristate acetate- and epidermal growth factor–induced COX-2 expression in carcinoma cells. This suppressive effect of retinoids may be mediated by AP-1 transrepression caused by competition for limited amounts of co-activators such as p300/CBP as suggested by Subbaramaiah et al. (40). Toxicity has limited the clinical use of retinoids that activate RAR-dependent pathways. Therefore, RXR-selective retinoids have been developed that do not activate RAR-dependent genes, and thus are much less toxic than retinoids that activate RAR-dependent pathways.

The current study suggests that rexinoids act in a similar manner by binding to RXR and inhibiting AP-1-dependent transcription. Whereas both the RXR-selective and the RAR-selective retinoids suppress COX-2 expression, the RAR-selective retinoids also activate other RAR-dependent genes, many of which lead to toxicity. The fact that rexinoids suppress COX-2 expression and inhibit cell growth as well or better than the RAR-selective retinoids, and yet are less toxic than RAR-selective retinoids makes them more attractive agents for the prevention of cancer.

Taken together with our previous results (19, 20), the current studies suggest that LGD1069 suppresses the development of breast cancer in part by suppressing COX-2 expression at the transcription level. LGD1069 inhibits AP-1-mediated induction of genes such as COX-2 and collagenase. RXR-selective retinoids that inhibit AP-1 activity and suppress COX-2 expression may be particularly promising drugs for molecular-based breast cancer prevention. Furthermore, such anti-AP-1 retinoids may be most useful in combination with other chemopreventive agents such as antiestrogens or COX-2 inhibitors for more effective prevention of breast cancer in women at high risk of this disease.

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The Retinoid X Receptor-Selective Retinoid, LGD1069, Down-regulates Cyclooxygenase-2 Expression in Human Breast Cells through Transcription Factor Crosstalk: Implications for Molecular-Based Chemoprevention

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