Induction of Adipocyte-specific Gene Expression Is Correlated with Mammary Tumor Regression by the Retinoid X Receptor-Ligand LGD1069 (Targretin)

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ABSTRACT

Targretin (LGD1069; a high-affinity ligand for the retinoid X receptors) is an efficacious chemotherapeutic and chemopreventive agent in the N-nitroso-N-methylurea-induced rat mammary carcinoma model. To evaluate the molecular action of LGD1069 in mammary carcinoma we have examined gene expression patterns in controls and nonresponding tumors compared with tumors undergoing regression (responding) by LGD1069. When compared with controls or nonresponding tumors, the expression of adipocyte-related genes such as adipocyte P2 (aP2), adipin, peroxisome proliferator-activated receptor γ (PPARγ), and lipoprotein lipase was elevated in LGD1069-responding tumors. Further analysis showed that gene expression changes occurred rapidly, in as little as 6 h, after the first dose of LGD1069. Immunohistochemical analysis showed that aP2 protein was also highly expressed in responding tumors when compared with control or nonresponding tumors. More importantly, aP2 protein was localized in the tumor cells in addition to the adipocytes present in the tumors. Similar changes in gene expression and inhibition in growth were seen in tumor cells (cloned from N-nitroso-N-methylurea-induced carcinoma) exposed to LGD1069 in vitro. These data suggest that tumor regression by LGD1069 involves differentiation induction along the adipocyte lineage.

INTRODUCTION

Breast cancer is the second leading cause of cancer death in women. One in eight women in the United States is at risk for developing breast cancer. Current adjuvant therapies include chemotherapy and hormone therapy, such as the antiestrogen TAM (1). Although TAM is highly efficacious, its use is limited by its side-effect profile, and, in general, no demonstrated additional efficacy is available after 5 years of therapy. More importantly, the majority of women will eventually fail TAM therapy (1–4). Thus, additional novel approaches to the treatment of breast cancer are necessary. The induction of breast cancer by the carcinogen NMU has served as an in vivo model to identify agents that suppress mammary carcinogenesis. Several classes of compounds have been identified that are highly efficacious in inhibiting mammary carcinogenesis. Two of these agents, antiestrogens and retinoids, inhibit both the incidence and number of NMU-induced tumors (5, 6).

Recently, we have demonstrated that LGD1069 (a selective ligand for the RXRs; Ref. 7) caused complete regression in 72% of NMU-induced rat mammary carcinomas (8). More importantly, LGD1069 also caused complete regression of tumors that previously failed TAM therapy (9). LGD1069 also has efficacy as a chemopreventive agent in the NMU-induced rat mammary carcinoma model that is equivalent to that of TAM (10). Mechanistically, LGD1069 causes regression of rat mammary tumors by inhibiting tumor cell proliferation (8). LGD1069 in vivo can also interfere with estrogen- or TAM-induced proliferation in the uterus (10). We have also observed in vitro that the RXR ligand LGD1069 inhibits the growth of human breast cancer cells and interferes with estrogen signaling and induces lipid accumulation in these cells.5

RXR ligands including LGD1069 have been shown to induce adipocyte-specific gene expression in preadipocytes and in human liposarcoma cells (Refs. 11, 12 and personal observation6). The expression of adipocyte-specific genes may be important in certain cancers, because an increase in aP2, adipin and PPARγ expression levels have been correlated with the inhibition of cellular proliferation in tumors of liposarcoma patients (13). In addition, the loss of aP2 expression has been shown to be associated with the progression of human bladder transitional cell carcinoma (14, 15). Therefore, to evaluate the effect of LGD1069 at the molecular level in responding rat mammary carcinomas, the expression of the adipocyte-related genes aP2, adipin, and PPARγ, and of genes encoding enzymes involved in fatty acid uptake and synthesis, including LPL (16), SCD1 (17), and FAS (18) was examined. A significant increase in expression of the adipocyte-related genes was observed in responding tumors when compared with control tumors or tumors that had failed LGD1069 therapy (nonresponders). Further analysis showed that aP2 protein was also highly expressed in responding tumors when compared with control or nonresponding tumors. Interestingly, aP2 protein was localized in the tumor cells in addition to the adipocytes present in the tumors. Similar changes in gene expression and inhibition in growth were seen in NMU417 cells (tumor cells isolated and cloned from an NMU-induced rat mammary tumor) exposed to LGD1069 in vitro. Hence, induction of adipocyte-related gene expression correlated with the regression of NMU-induced rat mammary carcinomas by LGD1069 treatment. Thus, tumor regression by LGD1069 involves differentiation induction along the adipocyte lineage.

MATERIALS AND METHODS

Reagents and Chemicals. All of the procedures were carried out under subdued ambient laboratory light. LGD1069 was synthesized at Ligand Pharmaceuticals, Inc. (San Diego, CA) and recrystallized to >99% purity. Powders were stored at −80°C prior to use.

Tumor Induction, Measurement, and Scoring. The procedures for tumor induction and measurement and scoring in the mammary gland of female Sprague Dawley rats were described earlier (8). In the present study, tumors were allowed to grow to a size of 300 mm3 in cross-sectional area prior to initiating oral administration of LGD1069. Animals were administered LGD1069, suspended in an aqueous solution composed of 10% (w/v) polyethylene glycol (M, 400)/Tween 80 (99.5:0.5) and 90% of 1% (w/v) carboxymethylcellulose (Sigma Chemical Co., St. Louis, MO) at 100 mg/kg (p.o., 7

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4 The abbreviations used are: TAM, tamoxifen; NMU, N-nitroso-N-methylurea; RXR, retinoid X receptor; LPL, lipoprotein lipase; aP2, adipocyte P2; PPARγ, peroxisome proliferator-activated receptor γ; SCD1, stearoyl-CoA desaturase-1; FAS, fatty acid synthase; RT, reverse transcription; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; RA, retinoic acid; RAR, RA receptor.
5 V. R. Agarwal, T. Hermann, R. P. Bissonnette, W. W. Lamph. The RXR-agonist LGD1069 (Targretin) inhibits cell proliferation and causes lipid accumulation in both PPARγ-positive and PPARγ-negative human breast cancer cells, manuscript in preparation.
6 P. J. A. Davies and W. W. Lamph, unpublished observations.
days a week), and the treatment continued until responding tumors showed at least two consecutive decreases in area totaling >40% from their maximum size. Progressively growing tumors (nonresponding tumors), grew over the course of treatment (their final area was at least 40% greater than their initial area) and were used to evaluate gene expression differences between the tumors that responded and the tumors that did not respond to LGD1069 treatment. These studies have a treatment period in the range of 20–60 days, and the growth of all of the tumors was monitored twice weekly throughout the course of the study. The last dose of LGD1069 was given 6 h prior to the killing of the animals. For statistical analysis of differences in gene expression levels, studies were performed using nine tumors of each category: control tumors, responding tumors, and nonresponding tumors.

**RNA Preparation,** RNA from rat tumors was prepared by using RNA STAT-60™, INC. (Friendware, TX). Poly(A)^+^ RNA was prepared by using oligotex, mRNA mini-kit (QIAGEN Inc., Santa Clarita, CA).

**Probes Used in RNase Protection Assays and Northern Blots.** For RNase protection probes, rat aP2 (GenBank accession no. U75581) and adipin (GenBank accession no. M90259) were amplified by RT-PCR. Amplified fragments were cloned into pGEM4Z and confirmed by sequencing. Specificity of the probes for rat sequences was verified by testing them against rat lung and brown fat RNA, known to contain both aP2 and adipin transcripts. A cDNA probe was synthesized by linearizing the aP2 and adipin constructs with BamHI and EcoRI, respectively, followed by in vitro transcription using T7 polymerase. For Northern blots, human LPL and mouse PPARγ cDNA probes were kindly provided by Dr. Johan Auwerx (U 325 INSERM, Institut Pasteur, F-59019 Lille, France) and Dr. Ronald M. Evans (Gene Expression Laboratory, The Salk Institute for Biological Studies, La Jolla California), respectively. Mouse SCD1 was amplified by PCR (provided by Dr. Deepak Lala, Ligand Pharmaceuticals, Inc. San Diego, CA; Ref. 17) Human FAS cDNA probe was provided by Dr. Marco Gottardis (Endocrine Oncology, Bristol-Myers Squibb Pharmaceutical Research Institute, Princeton, NJ).

**Northern Blot Analysis.** Poly(A)^+^ RNA (500 ng) was subjected to gel blot analysis by standard methods. Blots were hybridized with various probes labeled with [32P]dCTP using Ready To Go kit (Pharmacia Biotech, Uppsala, Sweden) according to the method of Church and Gilbert (20). Radioactive signals were quantified using a PhosphorImager (Molecular Dynamics) and normalized against GAPDH.

**Quantitative Real-Time PCR.** Quantitative analysis by Real-time PCR was performed according to the protocol of Perkin-Elmer. Total RNA (200 ng) in a final volume of 50 μl was transcribed into cDNA using murine leukemia virus reverse transcriptase and random hexamer primers according to the manufacturer’s protocol (Perkin-Elmer, Norwalk, CT). An additional reaction not containing the enzyme serves as minus-RT control. After synthesis, cDNA was diluted with water to 200 μl and frozen in 10-μl aliquots. Real-time PCR was performed using gene-specific primers and specific fluorescently labeled probes. Initial gel analysis of the final amplification reaction confirmed that the fluorescent signal resulted from the correct amplification product. In short, a 10-μl aliquot (10-ng RNA equivalents) of each cDNA was used for Real-time PCR in a final volume of 50 μl containing 300 ng of each forward and reverse primer and 100 μM of the fluorescent probe (according to the protocol supplied by Perkin-Elmer). Together with the samples and minus-RT controls, a serially diluted cDNA standard was analyzed in parallel. Reactions were performed in an ABI PRISM 7700 Real-time PCR machine (Perkin-Elmer) for 40 cycles of 15 s each at 95°C and of 60 s at 60°C. Quantitative analysis was performed using the threshold procedure (Perkin-Elmer protocol), and relative amounts were calculated from the standard curve. For all of the target genes analyzed, the corresponding minus-RT controls in all of the samples showed that the contribution of contaminating DNA to the signal was negligible. All of the target gene expression was normalized to the expression of the housekeeping gene 36B4 (21).

**Primers and Probes for Real-Time PCR.** The primers and probes used were as follows: Rat 36B4 (Ref. 21; GenBank accession no. X15096), Forward 5′-agactgcgtggtctcagggga-3′, Reverse 5′-ccctcaggggtcagggc-3′, and Probe JOE-amdmam cacttcgctagtcctc-tcctg-3′, and Probe 6FAM-tcctcaggggtcagggc-TAMRA; and Rat PPARγ (Ref. 22; GenBank accession no. AF156666), Forward 5′-ctggaggtc-tcaatcagctggat-3′, Reverse 5′-atggcttcgtcagctgca-3′, and Probe 6FAM-catttcagttggaggcceggc-TAMRA.

**Immunohistochemistry.** Affinity-purified rabbit antimiurine ALBP/aP2 (dilution 1:2000; Ref. 23) was a kind gift from Dr. David A Bernlohr (Biochemistry Department, College of Biological Sciences, University of Minnesota, St. Paul, MN). Tissues were fixed in zinc formalin (Anatech, Battle Creek, MI) for 10 h, embedded in paraffin, and sectioned on a sliding microtome at a thickness of 4 μm. Immunohistochemistry was performed according to the standard protocol of Pharmingen (San Diego, CA). Positive staining was visualized by incubating the slides in diaminobenzidine.

**NMU417 Cells.** The NMU417 cell line was isolated from an explanted NMU-induced rat mammary carcinoma at Ligand Pharmaceuticals by trypsin digestion and was grown in DMEM with 10% FBS. The explanted rat mammary tumor cells were clonied by plating in soft agar. Consistent with their malignant phenotype and their ability to grow in soft agar, NMU417 cells generated tumors when inoculated into nude mice (not shown).

**Thymidine Incorporation Assay.** The method used for the determination of the incorporation of radiolabeled thymidine was adopted from the procedure described by Shrivastav and Paulson (24). NMU417 cells were plated in 96-well plates at a density of 500 cells/well and were incubated with vehicle or LGD1069 for 4 days at 37°C. Subsequently, 1 μCi of [5-3H]thymidine (43 Ci/mmol; Amersham Pharmacia Biotech Inc., Piscataway, NJ) in culture medium was added to each well, and the cells were incubated for an additional 6 h. Cells were lysed, and incorporated [3H]thymidine was assayed according to the scintillation proximity assay protocol (Amersham Pharmacia Biotech Inc., Piscataway, NJ). Values represent the mean cpm of incorporated thymidine from triplicate wells.

**Anchorage-dependent Clonogenic Assay.** Clonogenic assays were performed as described previously (25). Briefly, NMU417 cells were plated (500 cells/100-mm dish) in triplicate and incubated with or without compound for 2 weeks. Colonies were stained with crystal violet and counted.

**Gene Expression Analysis.** For expression analysis, NMU417 cells were plated in 24-well plates at a density of 10,000 cells/well. LGD1069 (1 μM) or vehicle was added the following day, and cells were harvested in RLT-lysis buffer (QIAGEN Inc.) between 6 and 80 h after treatment. Total RNA was prepared using the RNeasy Mini-Kit (QIAGEN Inc.).

**Statistical Analysis.** Statistical significance of differences between two groups was determined by conducting a Student’s t test. P ≤0.05 was considered to be significant.

**RESULTS**

**Dynamics of Tumor Response to LGD1069 Therapy.** Previously, we have described the ability of LGD1069 to cause complete regression in 72% of primary NMU-induced rat mammary carcinomas (8). To evaluate the effects of LGD1069 on gene expression patterns in NMU-induced rat mammary tumors, three types of tumors were examined: control tumors (vehicle-treated), responders (tumors undergoing LGD1069 mediated regression), and nonresponders (tumors that failed LGD1069 treatment). Control tumors showed progressive growth throughout the experimental period. The growth kinetics of representative tumors from both responder and nonresponder categories are depicted in Fig. 1.

Analyses of RAR and RXR expression in NMU-induced mammary tumors indicate that these tumors express both RARα and RARγ and express high levels of RXRα with lower levels of RXRβ. The expression of RXRα was not changed after LGD1069 treatment, and the levels were similar in control, responding, and nonresponding tumors. However the expression of RXRβ was significantly increased in the responding tumors (not shown). Expression of RARβ was undetectable (not shown).

**LGD1069 Induces the Expression of Adipocyte-related Genes in Responding Tumors.** To evaluate whether treatment of NMU-induced mammary carcinomas with LGD1069 caused an increase of adipocyte differentiation in responding tumors, the expression levels
of the adipocyte-related genes *aP2*, *adipsin*, and *PPARγ* were determined in control, responding, and nonresponding tumors. Low levels of *aP2*, *adipsin* and *PPARγ* gene expression were observed in both vehicle-treated control and nonresponding tumors (Fig. 2, A and B). A significant increase of 8-fold ($P < 0.0003$), 2.8-fold ($P < 0.0003$), and 2.45-fold ($P < 0.008$) in the expression level of *aP2*, *adipsin*, and *PPARγ*, respectively, was observed in the tumors that responded to LGD1069 therapy when compared with control tumors (Fig. 2, A and B). Also, a significant increase of 5.8-fold ($P < 0.0004$), 3.7-fold ($P < 0.0004$), and 4.2-fold ($P < 0.0004$) in the expression level of *aP2*, *adipsin*, and *PPARγ* genes, respectively, was observed in responding tumors when compared with nonresponding tumors.

Furthermore, the expression level of genes encoding enzymes of fatty acid uptake and synthesis, including *LPL*, *FAS*, and *SCD1* were also examined. A 5-fold ($P < 0.001$) and 2.6-fold ($P < 0.02$) induction was observed in *LPL* and *FAS* gene expression level, respectively, in responding tumors when compared with control tumors (Fig. 2B). Also, a significant increase of 3-fold ($P < 0.001$) in *LPL* gene expression was observed in responding tumors when compared with nonresponding tumors. But the difference between the expression level of *FAS* in responding and nonresponding tumors was not significant (Fig. 2B). In contrast to the adipocyte-specific genes, the expression levels of *LPL* and *FAS* were significantly ($P < 0.04$) increased by 1.7- and 1.9-fold, respectively, in nonresponding tumors when compared with control tumors (Fig. 2B). No significant difference was observed in the expression level of *SCD1* either in the responding or nonresponding tumors when compared with the control tumors or between responding and nonresponding tumors.

In a separate experiment, naïve tumor-bearing animals were given a single oral dose (100 mg/kg) of LGD1069 6 h prior to harvesting the tumors for gene expression analyses. When compared with vehicle-treated control tumors, higher *aP2* expression levels were observed in 75% of LGD1069-treated tumors (Fig. 2C). The average *aP2* expression level in tumors from LGD1069-treated animals was 2.4-fold higher than the level in vehicle-treated control tumors.

**Histology and *aP2* Protein Expression in Mammary Carcinomas.** The histology of LGD1069-responding tumors showed an increase in stromal and surrounding tissue invasion as compared with vehicle-treated control tumors. Responding tumors also had a decreased number of cell layers and decreased cellular pleomorphism as indicated by a well organized, more differentiated phenotype than control tumors (Fig. 3). Morphologically, histologically and pathologically nonresponding tumors were similar to the vehicle-treated control tumors. In comparison to the control tumors a marked decrease in proliferating cellular nuclear antigen (PCNA) immunostaining was observed in the responding tumors (not shown). These results were similar to our previously published results for bromodeoxyuridine incorporation (8). No difference was observed between control and nonresponding tumors.

To evaluate whether LGD1069 induction of *aP2* gene expression within the tumor mass was attributable to adipocytes present in the tumor or was increased in the tumor cells themselves, the localization of the *aP2* protein in control and regressing tumors was examined. When compared with vehicle-treated control tumors, *aP2*-immunostaining was significantly increased in tumors undergoing LGD1069-mediated regression (Fig. 3). Intense *aP2*-immunostaining was observed in the adipocytes present in the stroma of regressing tumors, but more importantly, *aP2*-immunostaining was also observed within the tumor cells themselves (Fig. 3). Similar to control tumors, negligible immunostaining of *aP2* was observed in the nonresponding tumors.

**LGD1069 Caused a Decrease in Proliferation and an Increase in Adipocyte-related Gene Expression in Cloned Tumor Cells in Vitro.** To determine the effect of LGD1069 treatment on tumor cells, outside the complex tumor environment, tumor cells were cloned from an explanted rat mammary carcinoma by plating cells in soft agar.
Consistent with their malignant phenotype and their ability to grow in soft agar, NMU417 cells produced tumors when inoculated s.c. in nude mice (not shown). To determine whether the isolated tumor cells were responsive to LGD1069 treatment, cell growth kinetics were evaluated in the cloned tumor cells. The growth of NMU417 cells was evaluated in two separate assays, i.e., thymidine incorporation and anchorage-dependent clonogenic growth. Treatment with LGD1069 (1 μM) inhibited cellular proliferation of NMU417 cells by 77% (P < 0.0002; Fig. 4A). In clonogenic growth assays, colonies containing NMU417 cells were grouped mainly into two categories: large colonies containing 180–200 cells and small colonies containing 10–30 cells. LGD1069 (10^{-7} M) inhibited the formation of large colonies by 87.5% (P < 0.05) and small colonies by 58.8% (P < 0.02; Fig. 4B).

Because induction of adipocyte-related gene expression correlated with regression of NMU-induced rat mammary carcinomas by LGD1069, the expression of adipocyte-related genes aP2 and PPARγ was evaluated in NMU417 cells. The cells were treated with LGD1069 (1 μM) or vehicle over a period of 80 h and analyzed for aP2 and PPARγ expression levels. Both genes were dramatically increased by LGD1069 over the treatment period, whereas expression levels in vehicle-treated controls remained almost constant (Fig. 5).

**DISCUSSION**

These studies demonstrate that the increased expression of adipocyte-related genes aP2, adipsin and PPARγ correlate with LGD1069 mediated regression of rat mammary carcinomas. Importantly, aP2 protein was also highly expressed in regressing tumors when compared with control tumors and was localized in the tumor cells in addition to the adipocytes present in the tumor. In addition, LGD1069 inhibited the growth of and induced the expression of aP2 and PPARγ...
mRNA within a cloned tumor cell line (NMU417). Hence, LGD1069 seems to initiate a process of adipocyte differentiation in tumor cells leading to tumor regression.

The concept of differentiation induction therapy was described in 1927 in a report from Cushing and Wolbach (26) that during palliative treatment of an advanced childhood neuroblastoma, the metastases of this malignant tumor were found to have spontaneously differentiated into benign ganglioneuromas. More recently, the process of differentiation induction is a well-established paradigm for the treatment of some malignant diseases. Differentiation induction therapy with 1-β-D-arabinofuranosylcytosine (27), hexamethylene bisacetamide (28), or 5-azacytidine (29) has been shown to be effective in treating acute myelogenous leukemia; IFN-α treatment induced differentiation of hairy cell leukemia (30, 31). Of the differentiation inducing cancer therapeutics tested, RA derivatives (retinoids) have shown the most potential. Therapy with retinoids has demonstrated substantial clinical benefit in t(15;17) positive acute promyelocytic leukemia (32, 33) in juvenile chronic myelogenous leukemia (34) and T-cell lymphoma (35). Recently, we have observed the induction of adipocyte differentiation in clinical samples from liposarcoma patients after treatment with Targretin (LGD1069).6 Similarly, the induction of adipocyte differentiation in human liposarcoma after treatment with the PPARγ-ligand troglitazone has also been shown (13). In the present study, we demonstrate the induction of adipocyte differentiation in solid tumors of the mammary gland, or in mammary carcinomas by LGD1069 therapy. The process of adipocyte differentiation was characterized by the induction of adipocyte-specific gene expression and the loss of growth that correlated with the regression of carcinogen-induced rat mammary carcinomas.

Earlier, we demonstrated that the RAR and RXR pan-agonist 9-cis-RA induced cell differentiation that led to terminal cell division followed by cell death in the human promyelocytic leukemia cell line HL-60 (36). Similarly, in human breast cancer cell cultures, we have observed the induction of differentiation by lipid accumulation and inhibition of proliferation by the RXR ligand, LGD1069.5 In the NMU-induced rat mammary tumor model, we have also shown that LGD1069 inhibited the proliferation of tumor cells in vivo (8). Now, in the present study, we observed the expression of the adipocyte-specific protein aP2 in the tumor cells of regressing tumors during LGD1069 treatment. Additionally, we demonstrated the inhibition of cell growth and induction of adipocyte-related gene expression in tumor cells (NMU417) isolated and cloned from an explanted NMU-induced rat mammary tumor. Because we did not observe any morphological changes consistent with the aP2-expressing tumor cells differentiating into adipocytes, we are not implying that the tumor cells are undergoing transdifferentiation into mature adipocytes. We hypothesize that LGD1069 induces adipocyte differentiation within the tumor cells of NMU-induced mammary carcinomas that leads to terminal cell division followed by cell death.

The induction by LGD1069 of aP2 mRNA expression in rat mammary tumors occurs rapidly, in that when naive tumor-bearing animals were given a single oral dose of LGD1069, they showed increased aP2 expression in the tumors in as little as 6 h after administration. Interestingly, increased aP2 expression was not seen in all of the naive tumors exposed to LGD1069 but only in 75% of them. These numbers correlated well with our previous observation that LGD1069 causes complete regression in 72% of NMU-induced rat mammary carcinoma (8). This implies that the induction of aP2 expression may be an early predictive marker of responsiveness to LGD1069 therapy and may allow one to subset tumors into responders versus nonresponders based on the ability of LGD1069 to induce adipocyte-related gene expression.

The RXRs function as homodimers or as heterodimers with various nuclear receptors such as the PPARs, liver X receptors, farnesoid X receptors, vitamin D receptors, and thyroid hormone receptors (37). Because RXR ligands induced lipid accumulation and the expression of FAS gene in human breast cancer cell lines,5 and LGD1069 induced adipocyte-related gene expression in responding rat mammary tumors, it is possible that nuclear receptors involved in lipid homeostasis are the heterodimeric partners of RXR involved in the regression of NMU-induced tumors. RXR/PPARγ is a permissive heterodimer because ligands of either receptor activate this heterodimer in a variety of cells including preadipocytes (11). LGD1069 treatment induced PPARγ expression in NMU-induced mammary tumors responding to LGD1069. Thus, one possible heterodimer important in this model may be the RXR/PPARγ heterodimer, which is known to transcriptionally induce aP2 and PPARγ resulting in adipocyte differentiation (38). We and others (11, 12) have also shown that the activity of RXR/PPARγ is synergistically increased in preadipocytes when a RXR-ligand is combined with a PPARγ ligand. This synergistic activity may have implications for the treatment of mammary carcinoma. It will be interesting to examine the therapeutic effects of combining a RXR ligand with a PPARγ ligand in the NMU-induced rat mammary carcinoma model. Even when PPARγ

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**Fig. 4.** LGD1069 inhibits the in vitro growth of cells (NMU417) isolated and cloned from an explanted NMU-induced rat mammary carcinoma. In A, LGD1069 inhibits thymidine incorporation in NMU417 cells in a concentration-dependent manner. NMU417 cells were incubated with the indicated concentration of LGD1069 for 4 days and then pulsed for 6 h with [5-3H]thymidine. Each data point, the average ± SE of three experiments performed with triplicate wells. In B, LGD1069 inhibits the anchorage-dependent clonogenic growth of NMU417 cells. NMU417 cells were plated at 500 cells/100-mm dish and incubated with or without LGD1069 for 14 days prior to evaluating colony numbers. Results shown are the average ± SE of three individual experiments with triplicate dishes in each experiment.

**Fig. 5.** LGD1069 induces the expression of adipocyte-related genes aP2 (A) and PPARγ (B) in vitro in cells (NMU417) isolated and cloned from an explanted NMU-induced rat mammary carcinoma. NMU417 cells were treated with LGD1069 (1 μM) for up to 80 h. Expression was evaluated at the indicated time points and measured by Real-time PCR. Expression levels were normalized to 36B4 expression and are shown relative to the level in vehicle-treated control cultures at the 6 h time point. Each data point, the average ± SD of duplicate determinations from each sample.
expression in vehicle-treated control tumors is low, a single dose of LGD1069 induces aP2 expression; therefore, the RXR/PPARγ heterodimer may not be the only heterodimer important for the action of LGD1069 in the N-MU-carcinogenesis model. Other heterodimeric partners, e.g., liver X receptors, which are known to be involved in lipid homeostasis, may also play a role in LGD1069 action in N-MU-induced rat tumors.

From a therapeutic point of view, it was important to determine how to initiate or trigger the process of LGD1069-mediated regression in mammary tumors. This information may lead to the discovery of additional cancer therapeutic targets for RXR ligands. In the present report, we demonstrate that Targretin (LGD1069) induced adipocyte-specific gene expression in responding mammary carcinomas and that increased gene expression was correlated with the regression of these tumors. LGD1069 also inhibited the proliferation of N-MU-induced cloned tumor cells and induced adipocyte-related gene expression in cloned N-MU-induced mammary tumor cells in vitro. Thus, it appears that in the N-MU-carcinogenesis model, RXR ligands cause tumor regression by inducing adipocyte differentiation in the tumor cells, which is followed by terminal cell division and cell death.

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