

Antiproliferative Effect of Liver X Receptor Agonists on LNCaP Human Prostate Cancer Cells

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Abstract

Liver X receptors function as central transcriptional regulators for lipid homeostasis, for which agonists have been developed as potential drugs for treatment of cardiovascular diseases and metabolic syndromes. Because dysregulation of lipid metabolism has been implicated in sex hormone-dependent cancers, we investigated the effect of liver X receptor agonists on prostate and breast cancer cell proliferation. Treatment of human prostate cancer LNCaP cell lines with the synthetic liver X receptor agonist T0901317 decreased the percentage of S-phase cells in a dose-dependent manner and increased the expression of cyclin-dependent kinase inhibitor p27^{Kip-1} (p27). Knockdown of p27 by RNA interference blocks T0901317-induced growth inhibition, suggesting that p27 expression plays a crucial role in this signaling. Liver X receptor agonists also inhibited the proliferation of other prostate and breast cancer cell lines. The level of liver X receptor α expression correlated directly with sensitivity to growth inhibition by liver X receptor agonists. Retroviral expression of liver X receptor α in human breast cancer MDA-MB435S cells, which express low levels of endogenous liver X receptors and are insensitive to T0901317, sensitized these cells to T0901317. Consistent with our observations in LNCaP cells, T0901317 induces dramatic up-regulation of p27 in liver X receptor α -overexpressing MDA-MB435S cells. Furthermore, oral administration of T0901317 inhibited the growth of LNCaP tumors in athymic nude mice. Based on these results, modulation of the liver X receptor signaling pathway is a new target for controlling tumor cell proliferation; therefore, liver X receptor agonists may have utility as antitumor agents.

Introduction

Because of its androgen-dependent growth, prostate cancer has been treated with androgen deprivation therapies since Charles Huggins published his classic report more than 60 years ago (1). Initially, this type of therapy is effective on androgen-dependent prostate cancer, but within a few years, the disease usually recurs with the emergence of androgen-independent tumors. Because no effective treatment is available for hormone-refractory prostate cancer, a novel treatment for this disease is greatly needed. The normal prostate produces and secretes a significant amount of cholesterol in prostatic fluid. In benign prostatic hypertrophy and prostatic adenocarcinoma, the levels of tissue and secreted cholesterol are 2- to 10-fold higher than in healthy prostate (2, 3). It has also been reported that sterol response element binding proteins, transcriptional regulators that control the metabolic pathway of lipogenesis and cholesterol, are activated in androgen-independent tumors (4). Therefore, pathways controlling the maintenance of lipid and cholesterol homeostasis may

represent unexploited targets for the treatment of prostate tumors. Liver X receptors α and β , which are nuclear receptors activated by oxysterols such as 22(R)- and 24(S)-hydroxycholesterol, are emerging as a central transcriptional regulator for lipid homeostasis (5). Liver X receptors function as heterodimers with retinoid X receptors, and these dimers can be activated by ligands for either receptor. Liver X receptor α is expressed at high levels in liver, intestine, adipose tissue, and macrophages; whereas liver X receptor β , which was discovered in our laboratory, is expressed ubiquitously and was named ubiquitous receptor or UR (6). Liver X receptor response elements in liver X receptor target genes are direct repeats of the consensus AGGTCA separated by four nucleotides. Because both liver X receptors in macrophages control the cholesterol efflux pathway through the regulation of target genes including *ATP-binding cassette A1 (ABCA1)* and *apolipoprotein E*, synthetic liver X receptors agonists have been developed as antiatherogenic drugs (7). In this study, we have examined the effect of the synthetic liver X receptor agonist T0901317 (8) on the growth of a range of human sex hormone-dependent cancer cell lines and also investigated the *in vivo* effect using human prostate cancer LNCaP tumors grown in nude mice.

Materials and Methods

Materials. Synthetic nonsteroidal liver X receptor agonist *N*-(2,2,2-trifluoro-ethyl)-*N*-[4-(2,2,2-trifluoro-1-hydroxy-1-trifluoromethyl-ethyl)-phenyl]benzenesulfonamide (T0901317) was purchased from Alexis Biochemicals (San Diego, CA). 22(R)-Hydroxycholesterol and 24(S)-hydroxycholesterol were purchased from Steraloids (Newport, RI). A monoclonal anti-p27 antibody was from Transduction Laboratories (Lexington, KY). Polyclonal anti-Skp2 and anti-p21 goat immunoglobulin Gs were from Santa Cruz Biotechnology (Santa Cruz, CA). A monoclonal anti-actin antibody was from Chemicon (Temecula, CA). A monoclonal anti-c-Myc antibody 9E10 was prepared from hybridoma obtained from the American Type Culture Collection (Manassas, VA).

Cell Culture. Androgen-dependent LNCaP 104-S cells and androgen-independent LNCaP 104-R1 cells were maintained and cultured as described previously (9, 10). Human prostate cancer DU-145, PC-3, human breast cancer MCF-7, and MDA-MB435S cells were obtained from American Type Culture Collection and maintained in Dulbecco's modified Eagle medium supplemented with 10% fetal bovine serum.

Cell Proliferation Assay. Cell number was analyzed by measuring DNA content with the fluorescent dye Hoechst 33258 (Sigma, St. Louis, MO) as described previously (11).

Real-Time Quantitative Polymerase Chain Reaction. Total RNA was isolated using the TriZol Reagent (Invitrogen, Carlsbad, CA) and was treated with DNase I (DNA-free; Ambion, Austin, TX). Reverse transcription was performed with random hexamers and Moloney murine leukemia virus reverse transcriptase (Omniscript; Qiagen, Valencia, CA). The TaqMan primer/probe was designed using Primer Express (Applied Biosystems, Foster City, CA). The 5' end of the probe was labeled with reporter-fluorescent dye FAM. The 3' end of probe was labeled with quencher dye TAMRA. The sequences of primers and probes are as follows: ABCA1 primers, 5'-TGTCCAGTCCAG-TAATGGTTCTGT-3' and 5'-AAGCGAGATATGGTCCGGATT-3'; ABCA1 probe, 5'-ACACCTGGAGAGAAGCTTCAACGAGACTAACC-3'; sterol response element binding protein-1c primers, 5'-GGTAGGGCCAACG-GCCT-3' and 5'-CTGTCTTGGTTGTTGATAAGCTGAA-3'; sterol response element binding protein-1c probe, 5'-ATCGCGAGCCATGGATTGCAC-

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3'; p27 primers, 5'-CCGGTGGACCACGAAGAGT-3' and 5'-GCTCGC-CTCTTCCATGTCTC-3'; p27 probe, 5'-AACCCGGGACTTGGAGAAG-CACTGC-3', respectively. Real-time PCR was performed on an ABI PRISM 7700 system (Applied Biosystems) using the QuantiTect Probe PCR protocol (Qiagen). The rRNA Control kit (Applied Biosystems) was used to normalize transcript levels between samples.

Flow Cytometric Analysis. Cells were seeded at 5×10^5 cells in 6-cm dishes. After 3 days of culture in the presence or absence of T0901317, cells were collected and fixed in 70% ethanol/30% PBS overnight at -20°C . Fixed cells were washed with PBS, treated with 0.1 mg/mL RNase A in PBS for 30 minutes, and then suspended in 50 $\mu\text{g}/\text{mL}$ propidium iodide in PBS. Cell cycle profiles and distributions were determined using a BD FacsScan flow cytometer (BD Biosciences, San Jose, CA). Cell cycle distribution was analyzed using ModFit LT software (Verity Software House, Topsham, ME).

Western Blot Analysis. Protein extracts were prepared by lysing PBS-washed cells on the dish with Laemmli gel loading buffer without bromophenol blue dye. Protein concentration was determined with the Bradford reagent (Bio-Rad Laboratories, Hercules, CA) using a bovine serum albumin standard. Proteins were separated on 6% polyacrylamide gels containing SDS. Electrophoresis and blotting were performed as described previously (10). Measurement of actin expression was used as a loading control.

Ribonucleic Acid Interference Experiments. The RNA interference sequence was designed by using the AA scanning program from OligoEngine (Seattle, WA). DNA coding for an RNA interference for human p27 was prepared using the following oligonucleotides: 5'-GATCCCCGACTGCA-GAGACATGGAATTCAAGATTCATGTCTCTGCAGTGTCTTTTGGAAA-3' and 5'-AGCTTTTCCAAAAGCACTGCAGAGACATGGAA-TCTCTTGAATTCCATGTCTCTGCAGTGCAGGG-3'. These 64-mer oligonucleotides were annealed and ligated into the pH1RP vector (12). The p27-RNA interference expression plasmid was stably transfected into 104-S cells using Effectene (Qiagen) and selection for G418 resistance.

Retroviral Liver X Receptor α Expression. Ectopic expression of liver X receptor α was achieved by infecting MDA-MB435S cells with pLNCX2 retrovirus (Clontech, Palo Alto, CA) carrying the human liver X receptor α cDNA (13). Retrovirus was generated using the Phoenix-ampho packaging cell line (G. Nolan, Stanford University, Stanford, CA).

Athymic Nude Mice Study. Six- to 8-week-old male BALB/c *nu/nu* mice (NCI-Frederick, Frederick, MD) received subcutaneous injections (14) of 10^6 LNCaP 104-S cells suspended in 0.25 mL of Matrigel (BD Bioscience, Bedford, MA). Tumors were measured weekly using a caliper, and their volumes were calculated using the formula length \times width \times height \times 0.52 (14). The liver X receptor agonist T0901317 was administered by daily oral gavage in a sesame oil vehicle. Initial tumor volumes were $89.1 \text{ mm}^3 \pm 11.08$ (SEM) before treatment.

Data Analysis. Data are presented as the mean \pm SD or SEM of three experiments or are representative of experiments repeated at least three times.

Results and Discussion

To determine whether liver X receptor agonists inhibit human prostate cancer growth, we treated androgen-dependent LNCaP 104-S and androgen-independent LNCaP 104-R1 cells with 22(R)-hydroxycholesterol, 24(S)-hydroxycholesterol, and the nonsteroidal synthetic agonist T0901317. As shown in Fig. 1A, all three liver X receptor agonists inhibited the proliferation of both cell lines. T0901317 shows the most potent inhibition, possibly because of its high affinity for liver X receptor (8). Androgen-independent 104-R1 cells were somewhat more sensitive to this compound, compared with 104-S cells. The expression of liver X receptor-target genes was analyzed by real-time quantitative PCR. The expression of ABCA1 mRNA in androgen-independent 104-R1 cells was induced by T0901317 to the same level as in androgen-dependent 104-S cells (Fig. 1B). Another liver X receptor-target gene, *SREBP-1c*, which controls expression of various lipogenic enzymes (15), was also increased by T0901317 in 104-S and 104-R1 cells.

The effect of T0901317 on cell cycle distribution in the LNCaP sublines 104-S and 104-R1 was examined using flow cytometry of

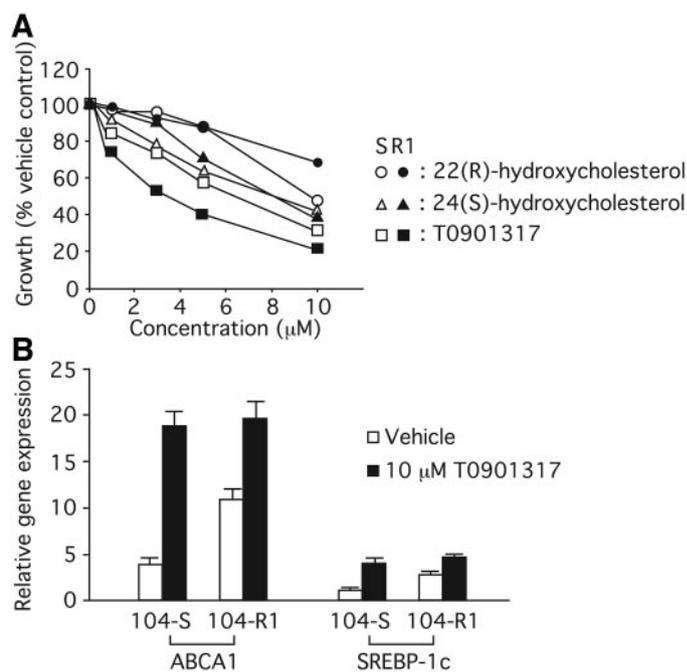
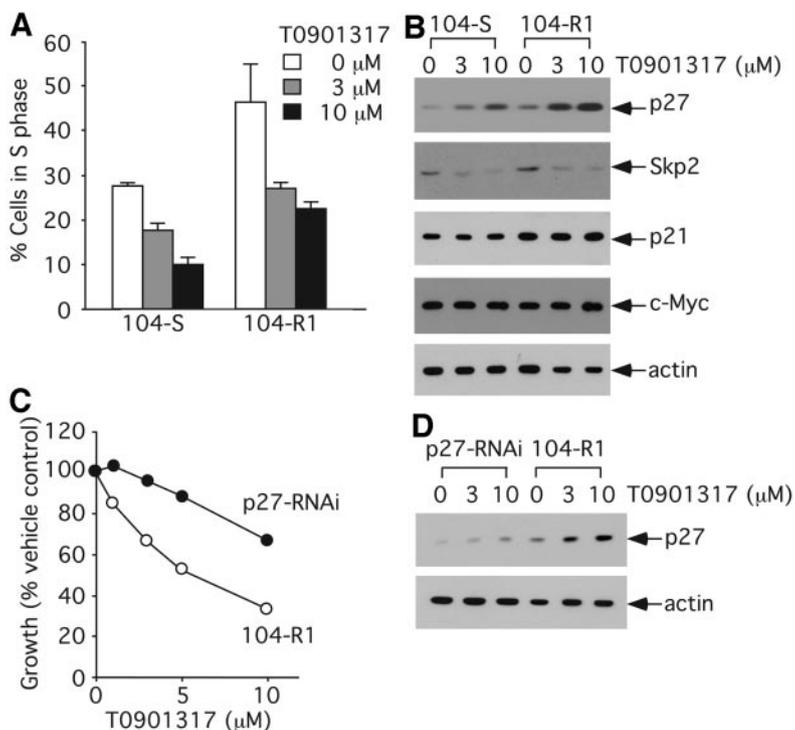


Fig. 1. Effect of liver X receptor agonists on proliferation and target gene expression in human prostate cancer LNCaP cells. *A*, effect of liver X receptor agonists on the proliferation of LNCaP sublines. 104-S and 104-R1 were grown for 4 days in the presence of 22(R)-hydroxycholesterol, 24(S)-hydroxycholesterol, and T0901317 at the indicated concentrations. Cell number was determined using a fluorometric DNA assay described in Materials and Methods. *B*, effect of T0901317 on expression of mRNA for liver X receptor target genes (*ABCA1*, *ATP-binding cassette transporter A1*; and *SREBP-1c*, *sterol regulatory element-binding protein-1c*). Cells were treated with vehicle or 10 $\mu\text{mol}/\text{L}$ T0901317 for 48 hours. mRNA levels were analyzed using real-time quantitative PCR. Gene expression was normalized to 18S rRNA content as described in Materials and Methods.

propidium iodide-stained cells. In both cell lines, T0901317 decreased the percentage of S-phase cells in a dose-dependent manner (Fig. 2A). The percentage of cells in the G_1 phase increased in proportion to decreases in S-phase cells (data not shown). Because we previously reported that the expression level of the cell cycle-dependent kinase inhibitor p27 is increased when LNCaP cells are arrested and in G_1 phase (10), Western blotting was performed to examine the effect of T0901317 on p27 expression. Consistent with the results of cell cycle analysis, T0901317 increased p27 in both LNCaP sublines (Fig. 2B). According to the real-time quantitative PCR analysis of p27 mRNA in T0901317-treated cells, there was no significant increase in p27 mRNA expression (data not shown), suggesting that the induction of p27 by T0901317 occurred by a posttranscriptional mechanism. T0901317 decreased the level of Skp2, a protein that mediates the ubiquitination and degradation of p27 (ref. 16; Fig. 2B). This change in Skp2 may account for the posttranscriptional induction of p27 by T0901317. The molecular mechanism responsible for the decrease in the level of Skp2 by T0901317 is currently under investigation. Other molecules involved in LNCaP cell proliferation (9, 10) were also analyzed by Western analysis. Neither c-Myc nor the cyclin-dependent kinase inhibitor p21 was influenced by T0901317 (Fig. 2B). To demonstrate that the level of p27 is functionally involved in T0901317-induced cell cycle arrest, we generated p27-knockdown 104-R1 cells using an expression plasmid generating RNA interference for p27. As shown in Fig. 2C, p27-knockdown 104-R1 cells were more resistant to T0901317 than 104-R1 cells. Western blotting confirmed the decrease in the level of p27 by RNA interference of p27 (Fig. 2D). These results suggest that p27 is an important mediator of liver X receptor agonist-induced growth inhibition in LNCaP cells.

The growth of various other breast and prostate cancer cell lines

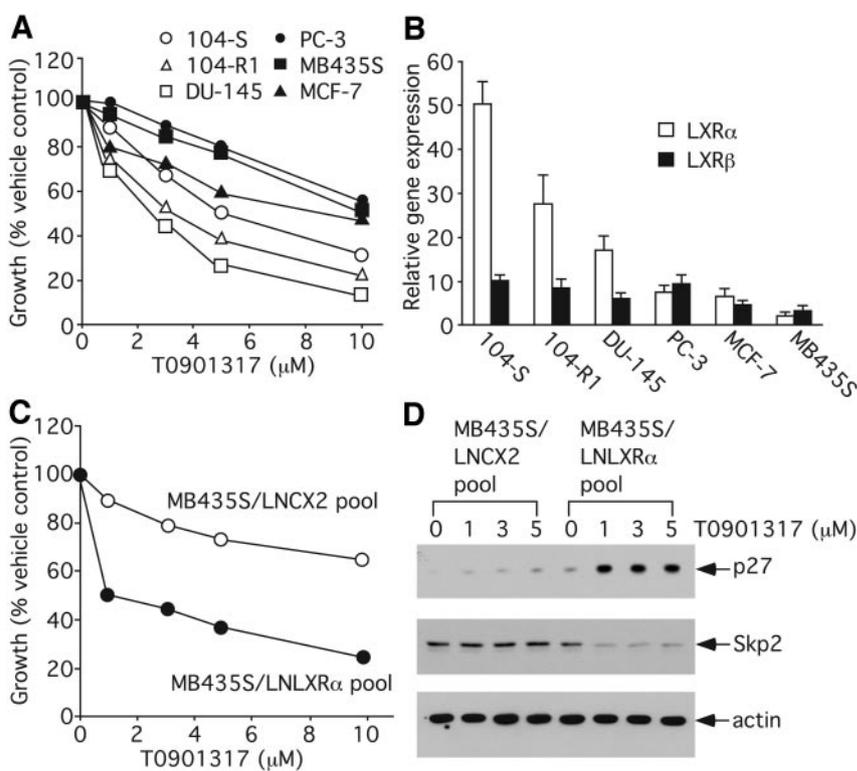
Fig. 2. Effect of liver X receptor agonist T0901317 on cell cycle and the expression of proliferation-related genes in LNCaP cells. **A**, percentage of 104-S cells and 104-R1 cells in S phase determined by flow cytometry of propidium iodide-stained cells. Cells were treated with vehicle or T0901317 for 3 days. **B**, effect of liver X receptor agonist on expression of p27, Skp2, p21, and c-Myc was analyzed by Western blotting. Cells were grown in the presence of T0901317 at the indicated concentrations for 3 days. **C**, growth of 104-R1 cells (○) and p27-knockdown 104-R1 cells (●). Cells were grown for 4 days in the presence of T0901317 at the indicated concentrations. Cell number was determined using a fluorometric DNA assay. **D**, expression levels of p27 in p27-knockdown cells was analyzed by Western blotting.



was also inhibited by T0901317 in a dose-dependent manner (Fig. 3A). In terms of sensitivity to T0901317, human prostate cancer PC-3 cells, breast cancer MCF-7, and MDA-MB435S cells were relatively insensitive to T0901317. Human prostate cancer LNCaP and DU-145 cells were more sensitive to this compound. Although the induction of ABCA1 by T0901317 in these cell lines did not show a significant relationship to sensitivity to T0901317, liver X receptor expression levels in the sensitive group were higher than in insensitive cell lines

(Fig. 3B). This suggests that other liver X receptor targets may be important. Using retroviral infection, we ectopically expressed human liver X receptor α in MDA-MB435S cells, which are relatively insensitive to T0901317 and have the lowest expression level of liver X receptors among these cell lines. The expression of liver X receptor α sensitized MDA-MB435S cells to T0901317 (Fig. 3C), suggesting that the expression levels of liver X receptor α is critical for T0901317-induced growth inhibition. Consistent with the results of the studies in

Fig. 3. Growth inhibition of various cancer cells by T0901317 and the expression levels of liver X receptors. **A**, effect of T0901317 on proliferation of various cancer cell lines. **B**, expression levels of liver X receptor α (□) and liver X receptor β (■) in the various cancer cell lines. Total RNA was isolated from cancer cells, and target gene transcripts were analyzed by real-time quantitative PCR. Gene expression was normalized to 18S rRNA content as described in Materials and Methods. For comparison of the expression levels of liver X receptor isoforms, the relative gene expression values are shown as relative to the value of liver X receptor α expression levels in MDA-MB435S cells. **C**, Retroviral expression of liver X receptor α in MDA-MB435S cells (●, MB435S/LNLXR α pool) enhanced growth inhibition by T0901317. ○, the control retroviral vector-infected cells (MB435S/LNCX2 pool). **D**, effect of retroviral expression of liver X receptor α in MDA-MB435S cells on the expression of p27 and Skp2. Retroviral infected cells were grown for 3 days in the presence of T0901317 at the indicated concentrations.



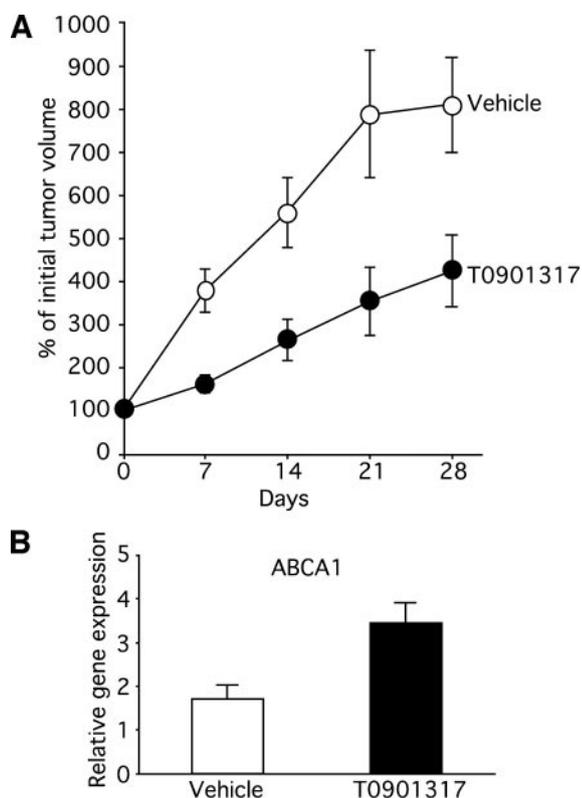


Fig. 4. Effect of T0901317 on tumor growth in male nude mice. A. LNCaP 104-S cells (10^6) were injected subcutaneously into both flanks of the mice. Four weeks later, tumor size was measured and designated as 100% on this (0th) day. For comparison, the size of tumors during the experimental period is expressed as a percentage of this initial tumor size. Values represent the mean \pm SEM of 10 mice, which were calculated on the basis of 13 to 15 tumors. Mice received 10 mg/kg T0901317 (●) or vehicle alone (○) by oral gavage once a day during the experiment period, resulting in a more than 2-fold difference in mean tumor growth between vehicle and T0901317-treated tumors after 28 days (Student's *t* test, $P = 0.0008$). B, the expression level of ABCA1 was analyzed by real-time quantitative PCR. At day 28, tumors were homogenized, and RNA was isolated by TriZol reagent and used for analysis. Values represent three animals from each group.

LNCaP cells (Fig. 2), T0901317-dependent induction of p27 and decrease in Skp2 were observed in these MDA-MB435S cells (Fig. 3D). These observations suggest that the liver X receptor-Skp2-p27 signaling pathway may mediate the antiproliferative activity of T0901317.

To determine whether liver X receptor agonists have antiproliferative effects *in vivo*, we tested T0901317 against LNCaP 104-S xenografts in athymic nude mice. T0901317 was administered via gavage using sesame oil vehicle at a dose of 10 mg/kg body weight per day. As shown in Fig. 4A, T0901317 significantly reduced the growth of LNCaP 104-S tumors. The activation of liver X receptor signaling by T0901317 in the tumors was confirmed by the induction of ABCA1 mRNA using real-time quantitative PCR analysis (Fig. 4B).

In the present study, we have shown that T0901317 inhibits cell cycle progression of prostate cancer cells. Inhibition of cell proliferation by T0901317 is correlated with liver X receptor expression levels, increases in p27, and decreases in Skp2, suggesting that the effect of T0901317 on proliferation may be mediated through the liver X receptor signaling pathway. Thus far, no cell cycle regulator or proliferation-related factors have been reported as liver X receptor target genes. However, Joseph *et al.* (17) demonstrated that the activation of liver X receptor antagonizes nuclear factor κ B signaling in macrophages, although the precise mechanism is unclear. Nuclear

factor κ B also plays an important role in cell growth control as a transcriptional up-regulator for cyclin D1 (18). However, based on Western blot analysis and reporter gene assays, we observed that liver X receptor agonists did not alter the expression level of cyclin D1 or transactivation by nuclear factor κ B in LNCaP cells (data not shown). It has also been reported that peroxisome proliferator-activated receptor γ agonists inhibit prostate cancer cells growth and decrease PSA levels in prostate cancer patients (19). Peroxisome proliferator-activated receptor γ agonists have been also used for treatment of other tumors. Because liver X receptor is a direct target of peroxisome proliferator-activated receptor γ (20), liver X receptor-induced growth arrest may explain the cell growth inhibition by peroxisome proliferator-activated receptor γ agonists. Our observations, therefore, may provide a new strategy for prostate cancer treatment through the modulation of liver X receptor target gene expression by liver X receptor ligands.

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