Antiproliferative Effect of LXR Agonists T0901317 and 22(R)-Hydroxycholesterol on Multiple Human Cancer Cell Lines

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Abstract. Previously, we and other groups reported that liver X receptor (LXR) agonists T0901317, 22(R)-hydroxycholesterol, and 24(S)-hydroxycholesterol suppressed the proliferation of prostate and breast cancer cells. In this study, we report that T0901317 and 22(R)-hydroxycholesterol treatment inhibited the proliferation of different progression stages of LNCaP human prostate cancer cells, as well as different commonly used human cancer cell lines. Cancer cell lines with higher LXRα mRNA expression were more sensitive to 22(R)-hydroxycholesterol-induced inhibition. T0901317 treatment decreased the percentage of the cell population in S-phase and caused G1 cell cycle arrest. Overexpression of S-phase kinase-associated protein 2 (Skp2) partially blocked the suppressive effect of T0901317 treatment. Modulating LXR signaling is therefore a potential adjuvant therapy for advanced prostate cancer and other types of cancer.

Liver X receptors (LXRs) are ligand-activated transcriptional factors that belong to the nuclear receptor superfamily. LXRs are important regulators of cholesterol, fatty acid, and glucose homeostasis (1). There are two LXR isoforms. LXRα expression is most abundant in liver, kidney, intestine, fat tissue, macrophages, lung, and spleen, while LXRβ is ubiquitously expressed (1). A specific group of oxysterols, such as 22(R)-hydroxycholesterol, 20(S)-hydroxycholesterol, 24(S)-hydroxycholesterol, and cholestenoic acid are natural ligands for LXRs (1, 2). A few potent synthetic LXR agonists have also been developed, including AT1-829 (3) and non-steroidal LXR agonists T0901317 (4) and GW3965 (5). LXR agonists have been developed as potential drugs for treatment of cardiovascular diseases and metabolic syndromes and are effective for treatment of murine models of atherosclerosis, diabetes, and Alzheimer’s disease (1, 3-5). Previously, we and other groups reported that synthetic LXR agonists (T0901317 and GW3965) and natural LXR agonists (22(R)-hydroxycholesterol and 24(S)-hydroxycholesterol) suppressed the proliferation of prostate and breast cancer cells in vitro (6-8). Treatment of athymic mice with T0901317 suppressed the growth of LNCaP prostate cancer xenografts (6). Gavage of T0901317 delayed the progression of androgen-dependent LNCaP tumors towards androgen independency in castrated athymic mice (9). LXR agonists appeared to cause G1 cell cycle arrest in LNCaP prostate cancer cells by reducing expression of S-phase kinase-associated protein 2 (Skp2), which resulting in the accumulation of cell cycle inhibitor p27Kip (6). In MCF-7 breast cancer cells, treatment with synthetic LXR agonists T0901317 and GW3965 suppressed the mRNA and protein expression of Skp2, cyclin A2, cyclin D1 and estrogen receptor (ER) α, while it increased the protein expression of p53 and decreased the phosphorylation of serine 780 and 795 of retinoblastoma (Rb) (7). Reduced phosphorylation at these two sites is related to an active form of the Rb protein that binds E2F and inhibits cell cycle progression. T0901317 is a very potent LXR agonist, its effective concentration to activate LXRα is 20 nM (4). On the other hand, 22(R)-hydroxycholesterol is a natural LXRα agonist which does not have cross-reactivity to other nuclear receptor (2). Our LNCaP prostate cancer progression model (9-13), in certain circumstances, mimics the clinical progression of prostate tumors from being hormone dependent to hormone refractory (14, 15). We thus investigated if T0901317 and 22(R)-hydroxycholesterol (2) can suppress different progression stages of LNCaP prostate cancer cells and other commonly used human cancer cell lines.

Materials and Methods

Materials. T0901317 was purchased from Alexis Biochemicals (San Diego, CA, USA). 22(R)-hydroxycholesterol was purchased from Steraloids (Newport, RI, USA).
Cell culture. LNCaP 104-S, 104-R1, 104-R2, R1Ad, CDXR-3 and IS-3 cells were passaged and maintained as described previously (9-13, 16, 17). PC-3, DU-145, A431, MCF-7, HeLa, MDA-MB-435, H1299, SCC13, HepG2, and Saos-2 cells were maintained in DMEM (Gibco/Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (Gibco/Invitrogen) and penicillin (100 U/ml) (Gibco/Invitrogen) and streptomycin (100 μg/ml) (Gibco/Invitrogen). All cancer cell lines were generous gifts from Dr. Shutsung Liao (Ben May Department for Cancer Research, The University of Chicago, Chicago, IL, USA).

Cell proliferation assay. Proliferation assays for LNCaP 104-S and R1Ad cells were performed in DMEM supplemented with 1 nM dihydrotestosterone (DHT) and 10% fetal bovine serum. For LNCaP 104-R1, 104-R2, CDXR-3, and IS-3 cells, proliferation assays were performed in DMEM supplemented with 10% dextran-coated charcoal stripped fetal bovine serum (CS-FBS). For other cancer cells, proliferation assays were performed in DMEM supplemented with 10% fetal bovine serum. Twenty four hours after plating cells, increasing concentrations of reagents were added to cells in an additional 100 μl medium and cell grown for another 96 hours. At the end of the experiment, the medium was removed by aspiration and 100 μl water was added into each well. The plate was kept at –80°C overnight and thawed at 37°C, followed by adding 100 μl of buffer containing10 mM Tris (pH 7.4), 1 mM EDTA, 2 M NaCl and 0.2% Hoechst 33258 (10 mg/ml stock) (Sigma, St. Louis, MO, USA). Relative DNA content was determined by measuring the fluorescence of Hoechst 33258 bound to DNA using a Wallac multilabel counter at 355 nm excitation and 460 nm emission wavelengths (Wallac/Perkin Elmer, Boston, MA, USA) (16,17).

Flow cytometric analysis. Cells were seeded at a density of 5x10^5 cells in 10 cm dishes in 10 ml of media and T0901317 (10 μM) was added 24 hours after plating. After 96 hours of culture in the presence or absence of T0901317, cells were removed with trypsin and fixed in 70% ethanol in PBS overnight at –20°C. Fixed cells were washed with PBS, treated with 0.1 mg/ml RNase A in PBS for 30 min, and then suspended in 50 μg/ml propidium iodide in PBS. Cell cycle profiles and distributions were determined by flow cytometric analysis of cells using a BD Facsan flow cytometer (BD Biosciences, San Jose, CA, USA). Cell cycle distribution was analyzed using ModFit LT software (Verity Software House, Topsham, ME, USA) as described.

Western blotting analysis. Protein extracts were prepared by lysing PBS-washed cells on the dish with 2x Laemmli gel loading buffer without bromophenol blue dye. Protein concentration was determined with the Bradford reagent (Bio-Rad Laboratories, Hercules, CA, USA) using a bovine serum albumin standard. Proteins were separated on 10% SDS-PAGE gels. S-phase kinase-associated protein 2 (p45) (Skp2) protein expression was determined by Western blotting using polyclonal anti-Skp2 rabbit immunoglobulin G from Santa Cruz Biotechnology (Santa Cruz, CA). Measurement of β-actin expression was used as a loading control (data not shown).

Skp2 overexpression in PC-3 cells and western blotting assay. Ectopic expression of Skp2 achieved by transfecting PC-3 cells with LPCX plasmid containing wild-type human Skp2 cDNA. Puromycin-resistant colonies were expanded and screened for increased Skp2 protein expression by Western blot analysis. PC-3 cells carrying empty LPCX vectors were used as controls. Both cell lines were generous gifts from Dr. Shutsung Liao. Cells were lysed in Laemmli buffer without bromophenol blue dye.

Transactivation assay. HEK293 cells were seeded at 3x10^4 cells/well in a 48-well plate in DMEM containing 10% FBS.

Table I. EC50 of T0901317 (A) and 22(R)-hydroxycholesterol (B) on growth inhibition in multiple human cancer cell lines.

<table>
<thead>
<tr>
<th>Cell line</th>
<th>T0901317 (μM)</th>
<th>Cell line</th>
<th>22HOC (μM)</th>
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<tbody>
<tr>
<td>HepG2</td>
<td>5.8</td>
<td>104-R2</td>
<td>4.7</td>
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<tr>
<td>MCF-7</td>
<td>7.1</td>
<td>CDXR-3</td>
<td>5.2</td>
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<tr>
<td>104-R2</td>
<td>7.5</td>
<td>104-S</td>
<td>5.7</td>
</tr>
<tr>
<td>IS-3</td>
<td>7.7</td>
<td>104-R1</td>
<td>5.8</td>
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<tr>
<td>104-R1</td>
<td>7.9</td>
<td>IS-3</td>
<td>5.9</td>
</tr>
<tr>
<td>CDXR-3</td>
<td>8.1</td>
<td>R1Ad</td>
<td>6.3</td>
</tr>
<tr>
<td>MDA-MB-435</td>
<td>10.0</td>
<td>DU-145</td>
<td>7.0</td>
</tr>
<tr>
<td>104-S</td>
<td>10.1</td>
<td>SCC13</td>
<td>7.2</td>
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<tr>
<td>SCC13</td>
<td>10.4</td>
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<td>7.4</td>
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<tr>
<td>DU-145</td>
<td>10.6</td>
<td>Saos-2</td>
<td>7.6</td>
</tr>
<tr>
<td>R1Ad</td>
<td>10.7</td>
<td>HeLa</td>
<td>7.7</td>
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<tr>
<td>Saos-2</td>
<td>13.4</td>
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<td>HeLa</td>
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<td>A431</td>
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</table>
Twenty-four hours after plating, HEK 293 cells were transfected with phRL-CMV-Renilla luciferase plasmid (1 ng/well), pSG5hRXRα and pSG5hLXRα (40 ng/well each), DR4-Fluc (40 ng/well), and Bluescript SKII+(750 ng/well) using the calcium phosphate co-precipitation method (16-18). Twenty-four hours after transfection, cells were treated with increasing concentrations of T0901317. After an additional 24 hours, cells were lysed in 50 μl passive lysis buffer (Promega, Madison, WI, USA) and luciferase activity was measured using a Dual-Luciferase kit (Promega) in a Monolight luminometer (BD Biosciences).

Data analysis. Data are presented as the mean ±SD of at least three experiments or are representative of experiments repeated at least three times. Student’s t-test (two-tailed, unpaired) was used to evaluate the statistical significance of results from proliferation assay experiments. An Excel add-in ED50V10 was used for calculating half maximal effective concentration (EC50).

Results

T0901317 and 22(R)-hydroxycholesterol suppress proliferation of LNCaP prostate cancer cell sublines. As determined by DR-4 reporter gene transactivation assay in HEK 293 cells transfected with LXRα, T0901317, 24(S)-hydroxycholesterol and 22(R)-hydroxycholesterol significantly activated LXRα at 30 nM, 300 nM, and 1 μM, respectively (Figure 1A). Due to the limited availability of 24(S)-hydroxycholesterol, we did not test its suppressive effect on different cancer cell lines. T0901317 (Figure 1B) and 22-hydroxycholesterol (22HOC) (Figure 1C) both dose-dependently suppressed the proliferation of all LNCaP cancer cell sublines (Figure 1, Table I). These results suggested that androgen-dependency and expression of androgen receptor (AR) level did not affect the growth inhibition caused by LXR agonists, thus LXR agonists may inhibit different progression stages of prostate tumors in patients.

T0901317 and 22(R)-hydroxycholesterol suppress proliferation of different human cancer cell lines. We next tested the effect of T0901317 (Figure 2A) and 22(R)-hydroxycholesterol (Figure 2B) on proliferation of commonly used human cancer cell lines, including androgen-insensitive AR-negative prostate cancer PC-3 and DU145 cells, estrogen-responsive estrogen receptor-positive breast cancer MCF-7 cells, hepatoma HepG2 cells, non-small lung cancer H1299 cells, cervical cancer HeLa cells, epidermoid carcinoma A431 cells, osteosarcoma saos-2 cells, melanoma MDA-MB-435 cells, and squamous carcinoma SCC13 cells. Overall, T0901317 suppressed cell proliferation at lower concentrations (Figure 2A, 2B), probably due to its higher affinity for LXRα (Figure 1A). However, 22(R)-hydroxycholesterol caused more proliferation suppression at 8 μM or higher concentrations (Figure 2, Table I). Oxysterols, such as 25-hydroxycholesterol and 7β-hydroxycholesterol, have been shown to suppress proliferation of HepG2 cells at 30 μM (19). It is possible that the oxysterol structure of 22(R)-hydroxycholesterol may cause additional suppressive effect at higher concentrations. Previously, we assayed the mRNA expression level of LXRα and LXRβ in six cancer cell lines (6). The expression levels of LXRβ were similar in these cell lines, while expression levels of LXRα were: LNCaP 104-S>104-R1>DU-145>PC-3=MCF-7>MDA-MB-435. We found
that cancer cell lines with higher LXRα mRNA expression were more sensitive to 22(R)-hydroxycholesterol treatment (Table I). This result suggested that activation of LXRα might be the main mechanism for 22(R)-hydroxycholesterol to cause cell growth inhibition. Compared to other human cancer cell lines, LNCaP sublines were relatively sensitive to 22(R)-hydroxycholesterol treatment. A431, H1299, PC-3, and HeLa cells were relatively more resistant to treatment with both T0901317 and 22(R)-hydroxycholesterol (Table I).

**T0901317 causes G1 cell cycle arrest in different cancer cells.** Previously we reported that treatment of LNCaP cell lines with T0901317 reduced the percentage of S-phase cells and the expression of Skp2, increased the expression of cyclin-dependent kinase inhibitor p27Kip, but had no effect on expression of c-Myc and p21Cip (6). Knockdown of p27Kip by RNA interference blocked T0901317-induced growth inhibition (6). We therefore tested if T0901317 also causes G1 cell cycle arrest in other human cancer cell lines. After 96 hours’ treatment, T0901317 reduced the population of cells in the S phase in PC-3, H1299, MCF-7, HepG2, and HeLa cells and increased the percentage of cells in the G1 phase (Figure 3A), therefore it caused G1 cell cycle arrest.

We did not observe a significant apoptotic cell population in either the control groups or the treatment groups. S-phase kinase-associated protein 2 (p45) (Skp2), a member of the F-box protein family, functions in the phosphorylation-dependent ubiquitination and down-regulation of p27Kip (20). T0901317 treatment caused decrease of Skp2 expression in all the above five cancer cell lines (Figure 3B). Overexpression of Skp2 in PC-3 cells partially blocked the suppressive effect of T0901317 (Figure 3B, 3C), suggesting that Skp2 and p27Kip are actually the targets for T0901317 in human cancer cells. Consistent with our previous observation (6), overexpression of c-Myc in PC-3 cells did not block the suppressive effect of T0901317 (data not shown).

**Discussion**

LXR agonists were reported to suppress the proliferation of prostate cancer (6, 9), breast cancer (6, 7), ovarian cancer (21) and leukemia (22) cells. Our study revealed that synthetic LXR agonist T0901317 and natural LXR agonist 22(R)-hydroxycholesterol also suppressed the proliferation of different progression stages of LNCaP human prostate cancer cell lines (Figure 1) as well as prostate cancer, breast...
cancer, hepatoma, non-small lung cancer, cervical cancer, skin cancer, osteosarcoma, and melanoma cells (Figure 2).

The EC\textsubscript{50} for 22(R)-hydroxycholesterol in suppressing the proliferation of cancer cells (Table I) is comparable to the concentration required for 22(R)-hydroxycholesterol to activate LXR\textalpha{} (1.5 μM) (2) (Figure 1A), this may explain why the level of LXR\textalpha{} correlates with the sensitivity of different cancer cells to 22(R)-hydroxycholesterol treatment (6)(Table I). The effective concentrations for 22(R)-hydroxycholesterol to suppress cancer cell growth is within the known physiological range of 22(R)-hydroxycholesterol in the human body and is much lower than the concentrations to activate other nuclear receptors (2). LXR\textbeta{}-ABCG1 signaling was reported to regulate sterol metabolism (23). Activation of LXR\textbeta{} inhibited the proliferation of T-cells but had no effect on cell viability (23). Since T0901317 did not inhibit the proliferation of CAOV3 ovarian cancer cells treated with siRNA against LXR\textalpha{} or LXR\textbeta{} (21), it is possible that 22(R)-hydroxycholesterol inhibited cell proliferation mainly through activation of LXR\textalpha{}, while inhibition of T0901317 may be caused by both LXR\textalpha{} and LXR\textbeta{} activation. We did not observe T0901317 to cause cancer cell growth inhibition at 300 nM (data not shown). It is unclear why the concentration needed for T0901317 to suppress the proliferation of human cancer cells is at least fifteen-fold higher than the effective concentration for T0901317 to activate LXR\textalpha{} (20 nM) (4). The concentration of T0901317 observed to cause suppression on proliferation of ovarian cancer cell lines by Scoles et al. was 10-50 nM when the researchers used 0.1% FBS (21). We used 10% FBS in our study, it is possible that some proteins or growth factors in serum may hinder the suppressive effect of T0901317.

Our current (Figure 3) and previous observations (6) indicate that LXR agonists suppresses Skp2, induces p27, and causes G1 cell cycle arrest in human cancer cells. Vedin et al. observed that treatment with LXR agonists also caused G1 cell cycle arrest in MCF-7 breast cancer cell lines. LXR agonists suppressed Skp2, cyclin A2, cyclin D1, but increased the expression of p53 and maintained the retinoblastoma (Rb) protein in a hypo-phosphorylated active form in MCF-7 cells (7). G1 Cell cycle arrest may be one of the main mechanisms for LXR agonists to suppress the proliferation of human cancer cells. Micro-Western Array (24), a powerful high-throughput western blotting assay, may help us to identify the signaling transduction pathway involved in proliferation suppression.

Figure 3. T0901317 inhibits cell cycle progression in different cancer cells. A: PC-3, H1299, MCF-7, HepG2, HeLa cells were treated with increasing dosage of T0901317 for 96 hours, harvested, and stained with propidium iodide dye for flow cytometric analysis for cell cycle distribution. Relative percentage of cells in S and G\textsubscript{1} phase in each group are shown. B: Skp2 protein expression of the above five cancer cell lines in control and T0901317 treated (10 μM, 96 hours) was compared by western blotting assay. β-Actin was used as loading control (data not shown). C: PC-3 cells overexpressing Skp2 with LPCX vector and control PC-3 cells (empty LPCX vector) were treated with increasing concentrations of T0901317 for 96 hours and analyzed by a 96-well proliferation assay. *Statistically significant difference (p<0.05) between the PC-3 control cells and PC-3 overexpressing Skp2- cells. D: Protein expression of Skp2 assayed by Western blotting in PC-3 LPCX control and PC-3 LPCX Skp2 cell lines.
caused by LXR agonists in cancer cells. In conclusion, LXR agonists T0901317 and 22(R)-hydroxycholesterol suppress the proliferation of multiple human cancer cell lines through induction of G1 cell cycle arrest. It is therefore possible to modulate LXR signaling as an adjuvant therapy for treatment of advanced prostate cancer and other types of cancer.

Acknowledgements

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Reference