LXR Agonists and ABCG1-dependent Cholesterol Efflux in MCF-7 Breast Cancer Cells: Relation to Proliferation and Apoptosis

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Abstract. Background: Liver X receptor (LXR) plays a key role in reverse cholesterol transport by inducing the expression of the ATP-binding cassette (ABC) transporters, implicated in cholesterol efflux. Recent data showed that LXR agonists inhibit the proliferation of multiple types of human cancer cells. However, whether these effects are related to cholesterol efflux has not yet been elucidated. Materials and Methods: Effects of two LXR agonists (TO901317 and 22(R)-hydroxycholesterol [22(R)-HC]) on proliferation, apoptosis and cholesterol efflux were examined in MCF-7 breast cancer cells. Results: Treatment with LXR agonists (TO901317 at 20 μM and 22(R)-HC at 2 μg/ml) inhibited proliferation and induced apoptosis of MCF-7 cells. Furthermore, LXR activation resulted in an increase in gene and protein levels of ABCG1 transporters and in cholesterol efflux to isolated high-density lipoprotein (HDL), without affecting the ABCA1/APOA-I mediated efflux. Under these conditions, a remarkable reduction of intracellular and membrane-associated cholesterol levels was observed. Conclusion: LXR activation in MCF-7 cells could deprive cells of cholesterol, required for their growth, by stimulating its efflux, resulting in the inhibition of cell proliferation and in stimulation of apoptosis.

Liver X receptor (LXR) is a transcription factor particularly expressed in the liver, intestine, adipocytes and macrophages. It belongs to the nuclear receptor superfamily and forms heterodimers with retinoid X receptor (RXR) (1). LXR was initially isolated from a human liver cDNA library as an orphan receptor, since its natural ligands were unknown (2). Natural derivatives of cholesterol, known as oxysterols, have since been identified as natural ligands of LXR (3). In addition to being activated by endogenous oxysterols, LXR can also be activated by synthetic agonists, such as TO901317. Activated LXR/RXR heterodimers induce genes involved in the regulation of cholesterol metabolism, including the ATP-binding cassette (ABC) transporters ABCA1 and ABCG1, which stimulate cholesterol efflux from cells to apolipoprotein A-I (APOA-I) and high-density lipoprotein (HDL) respectively. The use of LXR ligands, has led to a new pharmacological approach regarding atherosclerosis and cardiovascular diseases (4, 5).

In the past few years, anti-proliferative effects of synthetic and natural LXR agonists have been observed in various types of human cancer such as prostate (6-8), ovarian (9, 10) and breast cancer (11, 12) both in vitro and in xenograft models. These studies reported that LXR agonists suppress the proliferation of cancer cells through induction of G1 cell cycle arrest or by inhibition of the AKT survival pathway. However, the relationship between reduced tumoral growth and cholesterol efflux, an LXR-dependent step, remains unclear.

Human breast cancer MCF-7 cells have been reported to express LXR at the protein and mRNA levels (13) and it was shown that LXR activation leads to a reduction in the proliferation and an increase in p53 protein expression. We propose that the activation of LXR deprives cancer cell membranes of lipids essential for their growth, by stimulating cholesterol efflux. The aims of this study were: i) to investigate in MCF-7 breast cancer cells, if the eventual anti-proliferative effects of some LXR agonists is correlated with changes in expression of the pro-apoptotic gene BCL-2-associated X protein (BAX) and the anti-apoptotic gene B-cell lymphoma 2 (BCL-2), and ii) to determine if these effects are associated with changes in cholesterol efflux, in particular through expression of the ABC transporters. With
this aim, cells were treated with TO901317, a very potent synthetic LXR agonist, and with 22(R)-hydroxycholesterol [22(R)-HC], a natural LXR agonist that does not have cross-reactivity with other nuclear receptors (3).

Materials and Methods

Materials. Human breast cancer MCF-7 cells and human monocytic THP-1 cells were from the European Collection of Animal Cell Cultures (ECACC, Salisbury, UK). 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyltetrazolium bromide (MTT), TO901317, 22(R)-HC and phorbol 12-myristate 13-acetate (PMA), were purchased from Sigma Aldrich (Saint Quentin Fallavier, France). 7-Aminoactinomycin D (7-AAD) was obtained from BD Biosciences (San Jose, CA, USA). [1,2-3H]-cholesterol was from Perkin Elmer (Courtaboeuf, France). Aldrich (Saint Quentin Fallavier, France). Ecolite (+) Liquid Scintillation was purchased from MP Biomedicals (Illkirch, France). HDLs were isolated from fresh human plasma by ultracentrifugation. Apolipoprotein A-I (APOA-I) was a kind gift from Dr. Alan T. Remaley (NIH, Bethesda, MD, USA). Mouse antibody against ABCA1 was from Abcam (Cambridge, UK). Goat anti-mouse IgG-Horseradish Peroxidase (HRP) was obtained from Sigma Aldrich. Goat anti-rabbit IgG-HRP was from Santa-Cruz Biotechnology (Heidelberg, Germany). Uptilight US Blot HRP substrate was from Interchim (Montlucon, France). All other reagents were from Sigma.

Cell culture. MCF-7 cells and THP-1 cells were cultured at 37°C in a humidified incubator with 5% CO₂ in Dulbecco’s Modified Eagle’s Medium (DMEM) and Roswell Park Memorial Institute (RPMI) medium respectively, both supplemented with 10% fetal bovine serum (FBS), 1% glutamine and 1% penicillin-streptomycin. For experiments involving the use of LXR ligands, cells were incubated in serum-free medium containing 0.1% fatty acid-free bovine serum albumin (BSA). Prior to treatment with LXR agonists, THP-1 cells were differentiated into macrophage-like cells with 100 nM PMA for three days. Then the seeding medium was removed and cells were treated with LXR agonists (TO901317 at 20 μM or 22(R)-HC at 2 μg/ml), diluted in 0.1% BSA-containing medium for 24 h at 37°C. THP-1 cells were plated at a density of 1×10⁶ in a 6-well plate and were allowed to adhere overnight. THP-1 cells were treated for 24 h in a 96-well plate with LXR ligands. Cells were harvested with trypsin-EDTA (Sigma Aldrich) and homogenized. Cell death was estimated after DNA incorporation of fluorescent 7-AAD and fluorescence-activated cell sorting analysis using BD FACSArray (Le Pont de Claux, France). In this assay, cells with permeabilized plasma membrane were stained with 7-AAD. Data are presented as the percentage of 7-AAD-positive cells (7-AAD*).

Flow cytometric quantification of cell death. MCF-7 cells were treated for 24 h in a 96-well plate with LXR ligands. Cells were harvested with trypsin-EDTA (Sigma Aldrich) and homogenized. Cell death was estimated after DNA incorporation of fluorescent 7-AAD and fluorescence-activated cell sorting analysis using BD FACSArray (Le Pont de Claux, France). In this assay, cells with permeabilized plasma membrane were stained with 7-AAD. Data are presented as the percentage of 7-AAD-positive cells (7-AAD*).

RNA extraction and real-time quantitative Polymerase Chain Reaction (PCR). MCF-7 cells were plated at a density of 5×10⁵ in a 6-well plate and were allowed to adhere overnight. THP-1 cells were plated at a density of 1×10⁶ in a 6-well plate and were allowed to differentiate into macrophage-like cells with 100 nM PMA for three days. Then the seeding medium was removed and cells were treated with LXR agonists (TO901317 at 20 μM or 22(R)-HC at 2 μg/ml), diluted in 0.1% BSA-containing medium for 24 h at 37°C. Total RNA was isolated by the TriZol Reagent (Invitrogen, Cergy Pontoise, France), following the manufacturer’s instructions. The mRNA (1 μg) was then reverse-transcribed into cDNA using SuperScriptIII Reverse Transcriptase (Invitrogen). An initial denaturation step for 5 min at 70°C was followed by an elongation phase of 45 min at 50°C. Quantitative PCR was performed on a MyiQ2 Real-Time PCR Detection System (Bio-Rad, Marnes-la-coquette, France) using the SYBR Green Supermix. PCR was carried out for 45 cycles of 95°C for 30 s and 60°C for 30 s. The fluorescence was read during the reaction, allowing for continuous monitoring of the amount of PCR product. The values were normalized using β-actin as an endogenous internal standard. Relative quantification was performed using the ΔΔCT method. The sequences of the primers used are shown in Table I.

Table I. Primer sequences used in this study.

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Forward sequence</th>
<th>Reverse sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACTB</td>
<td>β-Actin</td>
<td>GCGAGTCCAGACGCAAG</td>
</tr>
<tr>
<td>BAX</td>
<td>BCL-2-associated X protein</td>
<td>AAAACACAGTCCAAGGCA</td>
</tr>
<tr>
<td>BCL-2</td>
<td>B-cell lymphoma 2</td>
<td>CCGACTCCACACACATGACC</td>
</tr>
<tr>
<td>ABCA1</td>
<td>ATP-binding cassette A1</td>
<td>TCCGACTCCCTGCCTGGCAATTA</td>
</tr>
<tr>
<td>ABCG1</td>
<td>ATP-binding cassette G1</td>
<td>GAAAGGGGAATGGAGAGA</td>
</tr>
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Western blot analysis. MCF-7 and THP-1 cells were plated and treated with LXR agonists in a 6-well plate, as described in the previous section on RNA extraction. Supernatants were then removed and cells were washed with fresh PBS and lysed in 200 μl RIPA buffer [50 mM Tris-HCl pH 8, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% Sodium Dodecyl Sulfate (SDS)], that contained the protease inhibitors aprotinin (2 μg/ml) and phenylmethylsulfonyl fluoride (PMSF) (1 mM). Lysates were centrifuged at 4°C for 30 min at 11400 xg. The protein concentration was determined using the BCA Protein Assay method (Sigma Aldrich). Proteins were separated with 4-15% SDS-PAGE.
polyacrylamide gel electrophoresis (SDS-PAGE) and were transferred to a nitrocellulose membrane. Membranes were blocked overnight with 5% milk Tris-buffered saline (TBS)-Tween solution at 4°C, then incubated for 2 h with primary antibodies against human ABCA1 (Abcam, ab18180) or ABCG1 (Novus Biologicals, NB400-132). A second 2-h incubation with a peroxidase-conjugated IgG secondary antibody was performed. Bands were visualized by Uplitlight US Blot chemiluminescent substrate kit (Interchim). The abundance of β-actin was used as control (monoclonal antibody from Sigma Aldrich).

Cholesterol efflux assay. MCF-7 cells were plated at a density of 10^5 cells per well in a 24-well plate in 500 μl of culture medium and allowed to adhere overnight. Cells were then incubated for 24 h at 37°C with 1 μCi/ml of [1,2-3H]-cholesterol in DMEM with 10% FBS. Then the labeling medium was replaced with DMEM containing 0.1% BSA to ensure a uniform distribution of the label among cellular pools, and cells were incubated for an additional 24 h. Then, cells were treated for 24 h with LXR agonists (TO901317 at 20 μM or 22(R)-HC at 2 μg/ml), diluted in 0.1% BSA-containing medium. Cholesterol efflux was performed overnight in serum-free DMEM containing HDL (50 μg/ml) or APOA-I (25 μg/ml). Finally, culture media were collected and cleared of cellular debris by a brief centrifugation, cells were harvested and cholesterol was extracted in isopropanol. Radioactivity was measured by liquid scintillation counting on a Hidex 300 SL instrument (Hidex, Turku, Finland). The cholesterol efflux was expressed as the percentage of the radioactivity released from the cells in the medium relative to the total radioactivity in cells plus medium.

Confocal microscopy. MCF-7 cells were plated at a density of 3×10^4 cells/cm^2 in Lab-Tek Chambered Coverglass (Nunc, Rochester, NY, USA) and were allowed to adhere overnight. Then the seeding medium was removed and cells were treated with vehicle control or LXR agonists for 24 h at 37°C. Cholesterol was localized using the Cholesterol Cell-Based Detection Assay Kit (Cayman Chemicals, Ann Arbor, MI, USA). Cells were fixed and washed according to the manufacturer’s instructions, and then they were incubated for 1 h with Filipin III (provided with the kit), which is a probe used for sterol staining and localization (14). Cells were analyzed with an inverted confocal Nikon A1RSi microscope.

Data analysis. Experiments were performed in triplicates and values correspond to the mean from at least three independent experiments. The Student’s t-test was used, and p-values of <0.05 were considered as being significant.

Results

TO901317 and 22(R)-HC inhibit growth and induce apoptosis of MCF-7 breast cancer cells. We determined the effects of TO901317 and 22(R)-HC on the viability of MCF-7 cells by MTT assays. As shown in Figure 1A, treatment with LXR agonists reduced the viability of MCF-7 cells by 30% and 37% (p<0.05) after 24 and 48 h, respectively, compared to the untreated cells. These results are in correlation with those obtained with cell death quantification by flow cytometric analysis after 7-AAD staining. 7-AAD is a cell nonpermeable DNA intercalating dye, used for dead cell discrimination. Indeed, the 24-h treatment with TO901317 and 22(R)-HC resulted in an increase of 7-AAD^+ cells from 5% in untreated cells to 17% and 15%, respectively (p<0.001) (Figure 1B).

To confirm whether the activation of apoptosis is involved in the LXR agonist-induced death of MCF-7 cells, the expression of pro- and anti-apoptotic genes was evaluated by real-time quantitative PCR. The results obtained showed a significant up-regulation of the pro-apoptotic gene BAX in treated cells (p<0.05), while the expression of the anti-apoptotic gene BCL-2 was slightly reduced (p<0.05) (Figure 2).
**LXR agonists stimulate cholesterol efflux in MCF-7 cells.** ABCA1 and ABCG1 are two well-known transporters that mediate cholesterol efflux to APOA-I and HDL respectively. These two genes are direct targets of LXR. Thus, we wanted to test the hypothesis that the effect of LXR activation on the proliferation of MCF-7 cells would be correlated with an induction of the extracellular efflux of cholesterol, and therefore a reduction in the cellular cholesterol content. We first evaluated the percentage of $[^3]$H-cholesterol efflux to HDL and APOA-I in the supernatants of cultured MCF-7 cells treated with LXR agonists, as described in the Materials and Methods section. Interestingly, treatment of MCF-7 cells with TO901317 and 22(R)-HC resulted in a significant increase of cholesterol efflux to HDL ($p<0.001$), whereas no significant changes were observed in APOA-I–mediated cholesterol efflux (Figure 3A).

In order to verify the cholesterol content in MCF-7 cells after LXR activation, cells were stained with the cholesterol marker Filipin III and were visualized under a confocal microscope. As expected, treatment of MCF-7 cells with synthetic LXR agonist TO901317 and 22(R)-HC resulted in a significant increase of cholesterol efflux to HDL ($p<0.001$), whereas no significant changes were observed in APOA-I–mediated cholesterol efflux (Figure 3B), thus confirming the results obtained from cholesterol efflux assays.

**LXR agonists induce ABCG1 but not ABCA1 expression in MCF-7 cells.** The expression levels of the transporters ABCG1 and ABCA1 were then, analyzed in MCF-7 cells by real-time quantitative PCR and western blot. Results showed an increase of ABCG1 mRNA and protein levels after treatment with LXR agonists (Figure 4A). ABCA1 gene expression had a very high threshold cycle value (Ct) in qPCR analysis (data not shown), which could reflect a low expression level of ABCA1 in MCF-7 cells. This was confirmed when we compared its expression with that of the monocytic cell line THP-1, a frequently used model of LXR activation which constitutively expresses the ABCA1 protein. Indeed, results showed a very low expression level of the ABCA1 gene and protein, in MCF-7 cells compared to THP-1, even when the cells were treated with LXR agonists (Figure 4B).

**Discussion**

The present study highlights the relation between the anti-proliferative and pro-apoptotic effects of LXR agonists and enhanced cholesterol efflux in MCF-7 breast cancer cells. We found that the synthetic LXR agonist TO901317 and the natural LXR agonist 22(R)-HC, both suppressed the proliferation, induced apoptosis and stimulated cholesterol efflux mediated by the ABCG1 transporter.

It is believed that activation of LXR in cancer cells causes G_s/G_1 cell cycle arrest. This is due to overexpression of the regulatory proteins p21 and p27 involved in the inhibition of cyclin/cyclin-dependent kinase (CDK) complexes that are necessary for cell cycle progression (6, 10-12), or the inhibition of the AKT survival pathway (8). However no data have been reported on the effect of LXR activation on the gene expression of pro-apoptotic BAX and anti-apoptotic BCL-2 in MCF-7 cells. We show here a reciprocal effect of LXR agonists on these two genes, suggesting an induced apoptosis, which is in agreement with our findings in the viability assay and the quantification of cell death.
The relationship between the effect of LXR on cholesterol efflux and its anti-proliferative role in breast cancer remains unclear. Epidemiological studies reported an association between high total cholesterol in the plasma and the prostate and breast cancer incidence (15). It was also demonstrated that increased plasma cholesterol levels in mice, submitted to a cholesterol-rich diet, was associated with mammary tumor formation (16). These observations suggest that plasma cholesterol may be involved in the control of breast cancer. However, no evidence for LXR implication in this process has been obtained. Tiwari and colleagues (17) recently revealed that targeting cholesterol-rich lipid microdomains in MCF-7 cells with methyl-beta-cyclodextrin, which is used to disrupt lipid rafts by removing cholesterol from membranes, led to an inhibition of pro-survival signaling and activation of pro-apoptotic pathways. It is also known that cholesterol-rich lipid rafts mediate the survival of prostate cancer cells via the AKT pathway (18) and that LXR activation suppresses this survival pathway and induces apoptosis and cholesterol efflux via ABCG1 (8). Our results on cholesterol efflux to isolated HDLs, in breast cancer MCF-7 cells, are consistent with these findings on prostate cancer cells. It was clear for us that cholesterol efflux was mainly enhanced through the ABCG1/HDL pathway rather than ABCA1/APOA-I efflux. The very low rate of ABCA1 expression in MCF-7 could explain why no changes were observed in the APOA-I cholesterol efflux assay. The mRNA expression of ABCA1 in MCF-7 cells was previously

Figure 3. Effect of Liver X receptor (LXR) agonists on cholesterol efflux in MCF-7 cells. A: [3H]-cholesterol efflux to high-density lipoprotein (HDL) (50 μg/ml) or APOA-I (25 μg/ml) of cultured MCF-7 treated with LXR agonists for 24 h, was performed overnight and calculated by liquid scintillation counting. B: Cells were incubated for 1 h with Filipin III, a sterol marker, and their fluorescence was then observed under an inverted Nikon A1RSi confocal microscope (see the Materials and Methods section). Data are the mean±SD of three different experiments. **p<0.001 versus untreated cells, using Student’s t-test.
investigated by Vedin and colleagues (11) who reported its induction after LXR activation. However, no data on the protein level were provided.

Our data raise the question of the role of LXR in cholesterol metabolism in breast cancer. Our hypothesis, that cholesterol deprivation of cancer cells through LXR-stimulated efflux towards the membrane, may inhibit cellular survival has been confirmed. Our findings suggest that LXR agonists are potent inhibitors of the supply of cholesterol which is required for progression of breast cancer cells. More details on the LXR modulation of cholesterol metabolism in breast cancer would be necessary to determine the importance of this pathway in anticancer therapy.

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References


