Liver X Receptor Activation Reduces Angiogenesis by Impairing Lipid Raft Localization and Signaling of Vascular Endothelial Growth Factor Receptor-2

Alessio Noghero, Alessia Perino, Giorgio Seano, Elisa Saglio, Giuseppe Lo Sasso, Franco Veglio, Luca Primo, Emilio Hirsch, Federico Bussolini, Fulvio Morello

Objective—Liver X receptors (LXR\(\alpha\), LXR\(\beta\)) are master regulators of cholesterol homeostasis. In the endothelium, perturbations of cell cholesterol have an impact on fundamental processes. We, therefore, assessed the effects of LXR activation on endothelial functions related to angiogenesis in vitro and in vivo.

Methods and Results—LXR agonists (T0901317, GW3965) blunted migration, tubulogenesis, and proliferation of human umbilical vein endothelial cells. By affecting endothelial cholesterol homeostasis, LXR activation impaired the compartmentation of vascular endothelial growth factor receptor-2 in lipid rafts/caveolae and led to defective phosphorylation and downstream signaling of vascular endothelial growth factor receptor-2 upon vascular endothelial growth factor-A stimulation. Consistently, the antiangiogenic actions of LXR agonists could be prevented by coadministration of exogenous cholesterol. LXR agonists reduced endothelial sprouting from wild-type but not from LXR\(\alpha^{−/−}/LXR\beta^{−/−}\) knockout aortas and blunted the vascularization of implanted angioreactors in vivo. Furthermore, T0901317 reduced the growth of LLC-1 tumor grafts in mice by impairing angiogenesis.

Conclusion—Pharmacological activation of endothelial LXRs reduces angiogenesis by restraining cholesterol-dependent vascular endothelial growth factor receptor-2 compartmentation and signaling. Thus, administration of LXR agonists could exert therapeutic effects in pathological conditions characterized by uncontrolled angiogenesis. (Arterioscler Thromb Vasc Biol. 2012;32:00-00.)

Key Words: angiogenesis ■ cholesterol ■ liver X receptor ■ vascular endothelial growth factor ■ lipid rafts

Liver X receptors \(\alpha\) and \(\beta\) (LXR\(\alpha\)/NR1H3, LXR\(\beta\)/NR1H2) are retinoid X receptor \(\alpha\)/NR2B1 heterodimers belonging to the nuclear hormone receptor superfamily. Through coordinate transcriptional actions, LXRs orchestrate cellular and systemic cholesterol homeostasis.\(^1\) LXR\(\alpha\) reduces cholesterol absorption, inhibit cholesterol synthesis, and favor cholesterol output via reverse transport and bile secretion. Endogenous LXR agonists, such as 22(R)-, 24-, and 25-hydroxycholesterol, are formed upon cholesterol loading of cells and mediate this physiological feedback loop.\(^2\) Furthermore, LXR activation with synthetic agonists (eg, T0901317, GW3965) exerts beneficial effects in atherosclerosis.\(^3\)\(^-\)\(^6\)

LXRs affect cell biology well beyond cholesterol metabolism. For instance, the antiatherosclerotic properties of LXR agonists also involve anti-inflammatory actions.\(^7\) Furthermore, LXR agonists can restrain the proliferation of several cell types by affecting cell cycle control and prosurvival pathways.\(^8\)\(^-\)\(^14\)

The precise mechanisms linking these complex effects to cholesterol homeostasis are largely unknown. One possibility is that LXR-dependent cholesterol mobilization may affect the structure and dynamics of specific membrane and vesicular compartments. Indeed, T0901317 has been shown to reduce the size and associated signaling of lipid rafts, cholesterol-enriched domains of the plasma membrane constituting fundamental signaling hubs for multiple transduction pathways.\(^13\)\(^,\)\(^15\)

Administration of LXR agonists can impair multicellular processes that involve angiogenesis, such as liver regeneration and tumor growth.\(^3\)\(^,\)\(^13\)\(^,\)\(^16\)

We and others have reported that endothelial cells express functional LXRs with LXR\(\beta\) representing the most important LXR isoform in this cell type.\(^17\)\(^-\)\(^19\)

Endothelial cells are particularly sensitive to cholesterol levels, given their wide membrane surface and richness in lipid rafts/caveolae. In line with this view, impairment of cholesterol homeostasis with itraconazole or cyclodextrin has been previously shown to affect vascular endothelial growth factor receptor-2 (VEGFR2, Flk-1/KDR) signaling in endothelial cells.\(^20\)\(^,\)\(^21\) Taken together, these findings imply that the tissue actions of LXR agonists may also involve so far unappreciated antiangiogenic effects.
Herein, we show that the pharmacological activation of endothelial LXRs is antiangiogenic as a result of synergistic reductions in cell migration, tubulogenesis, and proliferation. The antiangiogenic effects of LXR agonists involve endothelial cholesterol depletion and impaired VEGFR2 compartmentation and signaling. These findings highlight the potential for LXR-targeted therapeutic interventions in conditions of pathological angiogenesis.

Methods
A full description of experimental methods is provided in the online-only Data Supplement.

Gene Expression Analysis
RNA was extracted using affinity columns (Qiagen) or TRizol (Invitrogen). Microarray gene expression profiling was performed with HG-U133 Plus 2.0 microarrays (Affymetrix). For quantitative reverse transcription real-time polymerase chain reaction, mRNA levels were analyzed using the 2−ΔΔCt relative quantification system with 18S rRNA as the housekeeping gene. Gene silencing was performed with short hairpin RNA (shRNA) Mission RNA interference vectors (Sigma) against LXRβ (Target Set NM_007121) or scramble shRNA.

Cell Cholesterol
Lipids were extracted using chloroform/isopropanol/NP-40 (7:11:0.1). For homogenization and spinning, the organic phase was dried and suspended in a reaction mix containing cholesterol oxidase and a colorimetric probe (Biovision). Absorbance at 570 nm was finally measured in a microplate reader.

Immunofluorescence
Human umbilical vein endothelial cells (HUVECs) were fixed with 4% paraformaldehyde, blocked, and incubated with anti–caveolin-1 (Santa Cruz) and anti-VEGFR2 (R&D systems) antibodies, followed by incubation with secondary antibodies (Molecular Probes). Nuclei were counterstained with DAPI (Molecular Probes). Images were captured with a Leica AF6000 workstation equipped with a total internal reflection fluorescence module and analyzed with ImageJ software. Tumor sections were incubated with anti-ABCA1 (Novus Biological) or anti-CD31 (BD Biosciences) antibodies. Images were counterstained with DAPI (Molecular Probes). Images were acquired by using a Leica TCS SP2 AOBS confocal microscope and analyzed with Leica Confocal Software.

Cell Fractionation
For fractionation, cell lysates were adjusted to 45% sucrose by the addition of 90% sucrose and placed into ultracentrifugation tubes. A full description of experimental methods is provided in the online-only Data Supplement. Ten fractions were collected from the top SW-55Ti rotor (Beckman). Ten fractions were collected from the top SW-55Ti rotor (Beckman). Ten fractions were collected from the top SW-55Ti rotor (Beckman). Ten fractions were collected from the top SW-55Ti rotor (Beckman).

Migration and Tubulogenesis Assays
HUVEC migration was assayed in a Boyden’s chamber with a gelatin-coated polycarbonate membrane. The lower compartment of the chamber was filled with EBM-2 medium (Lonza) containing vascular endothelial growth factor-A (VEGF-A; 10 ng/mL). HUVECs were serum starved overnight in the presence of the indicated compounds and then added to the upper compartment of the chamber. After 8 hours of incubation, the membrane was fixed and stained for microscopic analysis.

In Vivo Neoangiogenesis
In vivo neovascularization was performed using a modified directed in vivo angiogenesis assay (Trevigen). On day 14, mice were euthanized and angioreactors were dissected. The vascularized basement membrane extract of each angioreactor was recovered and digested using CellSperse solution (Trevigen). Vessel-derived cells were pelleted, suspended in PBS, and counted by fluorescence-activated cell sorter analysis.

Data Analysis
Quantification of tubular structures was performed with WinRhizo Pro software (Regent Instruments). Quantification of immunofluorescence microphotographs was performed with ImageJ software (National Institutes of Health). Data are presented as average±SEM. Statistical significance was tested with unpaired Student t test or 1-way ANOVA with post hoc analysis. The number of corresponding experimental replicates is provided in each figure legend.

Results
LXR Activation Reduces HUVEC Cholesterol
Human endothelial cells express functional LXRs. Gene expression profiling by microarray (Figure IA in the online-only Data Supplement) and real-time polymerase chain reaction (Figure 1A–1E) indicated that treatment of HUVECs with the synthetic LXR agonist T0901317 modulated the mRNA expression of key genes involved in cholesterol trafficking: ATP-binding cassette subfamily A member 1 (ABCA1), ATB-binding cassette subfamily G...
member 1 (ABCG1), inducible degrader of the low-density lipoprotein receptor, cholesteryl ester transfer protein, and sterol regulatory element binding transcription factor 1c. The upregulation of these genes was blunted by shRNA-mediated silencing of LXRβ (Figures IB, IC, and II in the online-only Data Supplement), thus confirming that ABCA1, ABCG1, inducible degrader of the low-density lipoprotein receptor, cholesteryl ester transfer protein, and sterol regulatory element binding transcription factor 1c are bona fide LXR targets also in endothelial cells.

In line with transcriptional data, T0901317 caused a robust protein induction of cholesterol transporters ABCA1 and ABCG1 (Figure 1F), which mediate cholesterol efflux and reverse cholesterol transport.27,28 Instead, cholesteryl ester transfer protein and sterol regulatory element binding transcription factor 1c protein levels were only marginally affected by T0901317 in HUVECs. Accordingly, T0901317 reduced HUVEC cholesterol content dose-dependently (Figure 1G).

**LXR Activation Inhibits Endothelial Migration and Tubulogenesis**

Modifications in endothelial cholesterol balance and trafficking have been shown to affect key biological processes.21,29,30 To assess the impact of LXR activation on endothelial angiogenesis, we first examined the effect of different LXR agonists on VEGF-A–induced migration of HUVECs. LXR agonists GW3965 and T0901317 reduced migration dose-dependently compared with vehicle (Figure 2A). To rule out off-target effects of LXR-activating compounds, HUVEC migration was also evaluated in the background of LXR silencing. Instead, cholesteryl ester transfer protein and sterol regulatory element binding transcription factor 1c protein levels were only marginally affected by T0901317 in HUVECs. Accordingly, T0901317 reduced HUVEC cholesterol content dose-dependently (Figure 1G).

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LXR Activation Inhibits Endothelial Proliferation Without Affecting Apoptosis

Besides motility, angiogenesis involves endothelial proliferation. As LXR agonists have been shown to reduce cell cycle progression in nonendothelial cells,8–11,14 we assessed the effect of LXR agonists on HUVEC proliferation. GW3965 and T0901317 reduced HUVEC proliferation dose-dependently (Figure 3A). A similar effect was also produced by natural LXR agonist 22-hydroxycholesterol (Figure IIIA in the online-only Data Supplement). Tubulogenesis was unchanged by T0901317 when HUVECs were coincubated with exogenous cell-soluble cholesterol, thus suggesting that LXR activation impairs in vitro angiogenesis by affecting cell cholesterol homeostasis (Figure 2C; Figure IIIA in the online-only Data Supplement). Taken together, these findings suggested that inhibition of endothelial migration and morphogenesis by LXR agonists is mediated by LXR-specific effects on cell cholesterol homeostasis.
the G1–S cell cycle transition, as assessed by propidium iodide staining (Figure 3D), and reduced the DNA incorporation of a thymidine analog (Figure 3E). The effect of T0901317 on G1–S transition and DNA synthesis was relieved by coincubation of HUVECs with exogenous cell-soluble cholesterol (Figure 3D and 3E), thus indicating that LXR activation impairs endothelial cell cycle and proliferation by affecting cell cholesterol homeostasis.

LXR Activation Reduces Signaling of VEGFR2
The observed effects of LXR agonists in endothelial cells suggested the involvement of LXR in the angiogenic signaling. As previously reported in macrophages, T0901317 upregulated the mRNA expression of VEGF-A also in HUVECs (Figure IVA in the online-only Data Supplement).33 However, the protein levels of VEGF-A were unchanged by T0901317 in HUVECs (Figure IVB in the online-only Data Supplement). The mRNA and protein expressions of VEGFR2, the pivotal VEGF receptor controlling angiogenesis,34 were not affected by T0901317 (Figure IVC in the online-only Data Supplement and Figure 4A). However, treatment of HUVECs with T0901317 blunted the functional activation of VEGFR2 by VEGF-A, as demonstrated by reduced phosphorylation of VEGFR2 on Tyr1175 and by reduced phosphorylation of phospholipase Cγ, a key mediator of VEGFR2-dependent angiogenic responses (Figure 4A).34 VEGF-A–dependent phosphorylation of VEGFR2 and phospholipase Cγ was unchanged by T0901317 when HUVECs were coincubated with exogenous cell-soluble cholesterol (Figure 4B and 4C), suggesting that LXR activation restrains VEGFR2 signaling by affecting cell cholesterol homeostasis. In line with this finding, the inhibitory effect of T0901317 on VEGF-A/VEGFR2–driven chemotaxis was specifically attenuated by the addition of exogenous cell-soluble cholesterol (Figure IVD in the online-only Data Supplement). On the contrary, T0901317 treatment did not affect the migration of HUVECs stimulated by fibronectin in a haptotaxis assay (Figure IVE in the online-only Data Supplement).

LXR Activation Reduces Compartmentation of VEGFR2 in Lipid Rafts/Caveolae
Cholesterol depletion by T0901317 has been associated with a decrease in lipid raft size and signaling.11 We thus tested the hypothesis that T0901317 may affect VEGFR2 signaling by impairing its compartmentation in lipid rafts/caveolae. We analyzed the cell surface distribution of caveolar rafts, visualized by caveolin-1 staining, and of VEGFR2, by total internal
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reflection fluorescence microscopy. Treatment of HUVECs with T0901317 reduced the caveolin-1–positive area by 53±8% (P<0.05) (Figure 4D and 4E), indicating a depletion in endothelial lipid rafts/caveolae. Furthermore, the amount of VEGFR2 localized in caveolar structures was reduced by 54±7% (P<0.05) (Figure 4D and 4F). The effect of T0901317 on caveolin-1 and VEGFR2 compartmentation was blunted by the addition of exogenous cell-soluble cholesterol (Figure 4E and 4F). Membrane fractionation experiments confirmed that T0901317 displaced VEGFR2 from the buoyant flotillin-2–positive and caveolin-1–positive fractions (1–3), representing endothelial lipid rafts/caveolae. Instead, the amount of VEGFR2 in the heavier, binding immunoglobulin protein–positive fractions, representing the endoplasmic reticulum, was not affected (Figure 4G and 4H). Coincubation of HUVECs with exogenous cell-soluble cholesterol attenuated the T0901317-dependent depletion of VEGFR2 in lipid rafts/caveolae (Figure 4I), indicating that LXR activation modifies VEGFR2 membrane compartmentation by affecting cell cholesterol homeostasis.

LXR Activation Inhibits Endothelial Sprouting and In Vivo Neoangiogenesis

The impact of LXR agonists on angiogenesis was next assayed ex vivo and in vivo. Incubation of mouse aortic rings with GW3965 or T0901317 reduced ex vivo endothelial sprouting by 89±2% and 73±10%, respectively, compared with vehicle (P<0.05). However, endothelial sprouting was unchanged by T0901317 when aortic rings were coincubated with cholesterol, indicating that LXR activation impairs endothelial sprouting through cholesterol depletion (Figure 5A). T0901317 reduced endothelial sprouting when applied to aortic rings obtained from wild-type mice but not to rings obtained from mice deficient for LXRs (LXRα−/−/LXRβ−/−) (Figure 5B), thus confirming that the antiangiogenic effect of T0901317 is LXR-specific. In vivo neoangiogenesis was next assessed through the subcutaneous implantation in mice of angioreactors containing VEGF-A and fibroblast growth factor–enriched extracellular matrix. Addition of T0901317 to the extracellular matrix critically reduced neoangiogenesis by 86±7% compared with vehicle (P<0.05) (Figure 5C), as assessed by red blood cell content in the angioreactors 2 weeks after implantation. Taken together, these data indicated that LXR agonists can inhibit angiogenesis in mouse tissues.

LXR Activation Inhibits Tumor Angiogenesis

Neoangiogenesis is strictly required for tumor growth. To highlight the effect of LXR activation on cancer angiogenesis, we used LLC-1 tumor grafts. Contrary to HUVECs, LLC-1 cells did not regulate the protein levels of cholesterol transporters ABCA1 and ABCG1 upon treatment with T0901317 (Figure 6A). Also, the levels of low-density lipoprotein receptor were unchanged by T0901317 in LLC-1 cells. Of note, T0901317 did not modify the proliferation (Figure VA and VB in the online-only Data Supplement), p27/Kip-1 expression (Figure VC in the online-only Data Supplement), and apoptosis (Figure VD in the online-only Data Supplement) of LLC-1 cells in vitro. LLC-1 cells were thus injected into the flank of wild-type mice, where they gave rise to palpable tumors within 7 days. Starting on day 7 after injection, mice were treated with T0901317 or vehicle daily (20 mg/kg IP) for 1 week. Administration of T0901317 significantly reduced...
tumor growth by 62±20% on day 7 compared with vehicle (P<0.05) (Figure 6B). Body weight remained similar in treated and control animals (data not shown). Administration of T0901317 upregulated ABCA1 mRNA within the tumor mass (Figure VE in the online-only Data Supplement). In particular, T0901317 significantly increased the protein expression of ABCA1 selectively within tumor endothelial cells (Figure 6C). Of note, T0901317 treatment reduced the endothelial density of LLC-1 tumors by 50±3% compared with vehicle (P<0.01), as assessed by CD31 immunostaining of tumor sections (Figure 6D). Taken together, these findings indicated that pharmacological targeting of LXRs can significantly reduce tumor angiogenesis.

Discussion

We provide evidence that LXR activation can restrain angiogenesis. The antiangiogenic effects of LXR agonists are strictly connected to their impact on endothelial cholesterol homeostasis, because coincubation of HUVECs or aortic rings with exogenous cholesterol largely neutralized the actions of LXR agonists on endothelial tubulogenesis, sprouting, and proliferation. These results are in line with previous reports that drugs leading to cholesterol deprivation or impaired cholesterol trafficking, such as β-cyclodextrin, itraconazole, or high-dose statins, can negatively affect angiogenesis. 29,30,35 In endothelial cells, T0901317 lowered cell cholesterol by regulating the expression of multiple targets. In particular, LXR activation increased the protein levels of cholesterol transporters ABCA1 and ABCG1, which promote cholesterol output toward apolipoprotein A–containing lipoproteins, as well as transendothelial high-density lipoprotein traffic.28,36 T0901317 also increased the mRNA expression of inducible degrader of the low-density lipoprotein receptor, which targets low-density lipoprotein receptor to ubiquitin-mediated

Figure 4. Liver X receptor (LXR) activation impairs vascular endothelial growth factor receptor-2 (VEGFR2) signaling and compartmentation in lipid rafts/caveolae. A, Protein levels of phospho-VEGFR2 (p-VEGFR2), total VEGFR2, and phospho-phospholipase Cγ (PLCγ) in human umbilical vein endothelial cells (HUVECs) treated with VEGF-A (30 ng/mL, 5 minutes) after incubation for 18 hours with vehicle (dimethyl sulfoxide [DMSO]), T0901317 (T090, 10 µmol/L), and cholesterol (CHOL, 20 µmol/L). Representative blots of 4 experiments. B, Quantification of P-VEGFR2 in HUVECs treated as in A. ***P<0.001 vs DMSO, †P<0.05 vs T090+CHOL, n=4. C, Quantification of P-PLCγ in HUVECs treated as in A. ***P<0.001 vs DMSO, ††P<0.01 vs T090+CHOL, n=3. D, Immunofluorescence staining of HUVECs treated for 18 hours with vehicle (DMSO), T0901317 (10 µmol/L), and cholesterol (20 µmol/L). Representative images of total internal reflection fluorescence (TIRF): red (caveolin-1), green (VEGFR2), blue (DAPI), yellow (merge caveolin-1/VEGFR2). E, Quantification of cell surface caveolin-1–positive area. *P<0.05 vs DMSO, †††P<0.001 vs T090+CHOL, n=20. F, Quantification of cell surface VEGFR2 in caveolin-1–positive areas. **P<0.01 vs DMSO, †††P<0.001 vs T090+CHOL, n=20. G to I, Distribution of VEGFR2, flotillin-2 (Flot-2), caveolin-1 (Cav-1), and binding immunoglobulin protein (BiP) in the protein fractions obtained from HUVECs (ultracentrifugation on a discontinuous sucrose gradient) treated as in D. The lipid raft compartment is represented in the lighter fractions (1–3), whereas the endoplasmic reticulum peaks in the heavier fractions (7–10). Representative blots of 3 experiments.
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A reductase, mevalonate kinase, and squalene epoxidase.19 Cholesterol biosynthesis, such as 3-hydroxy-3-methylglutaryl-coenzyme A reductase, mevalonate kinase, and squalene epoxidase.19 hence, natural LXR agonist 22(R)-hydroxycholesterol downregulates the expression of several genes controlling cholesterol biosynthesis, such as 3-hydroxy-3-methylglutaryl-coenzyme A reductase, mevalonate kinase, and squalene epoxidase.19 Hence, natural oxysterol LXR ligands may further impact on endothelial cholesterol homeostasis through synergistic effects on cholesterol synthesis and traffic. Nonetheless, we cannot exclude that antiangiogenic effects of LXRs may also relate to so far unappreciated perturbations of the nonsterol branch of the mevalonate pathway. For instance, LXR agonists may modify the synthesis of isoprenoids and affect protein prenylation, which is required for proper compartmentation and function of key proteins involved in angiogenesis (eg, small GTPases).

Cholesterol is essential to maintain a normal structure and function of cellular membranes. Furthermore, cholesterol-rich microdomains of the plasma membrane called lipid rafts/caveolae function as preferential sites for membrane receptor clustering and signaling.15 LXR activation has been previously shown to reduce lipid raft size and restrain lipid raft-associated signaling in a cancer cell line.13 We now provide evidence that in endothelial cells, treatment with LXR agonist T0901317 specifically impairs biological signals stemming from lipid raft/caveolar domains that are critical for angiogenesis. In particular, LXR activation (1) impaired VEGFR2 phosphorylation and downstream signaling to phospholipase Cγ, and (2) blunted the compartmentation of VEGFR2 in lipid rafts/caveolae. Both effects appear to be mediated by the LXR-dependent perturbation of cholesterol homeostasis. These findings are in line with previous data showing that changes in endothelial cholesterol affect VEGFR2 signaling, most likely as a result of complex interactions of the receptor and its signaling platform within lipid rafts/caveolae.20,21 Nonetheless, the molecular circuitry linking cholesterol balance to angiogenesis is only beginning to emerge. In our study, LXR activation significantly reduced caveolin-rich regions of the plasma membrane. As knockout of caveolin-1 inhibits angiogenesis by impairing VEGFR2 compartmentation and by causing a detrimental deregulation of endothelial NO synthase, LXR activation may restrain angiogenesis by altering the physiological conditions of caveolin-1 expression and internalization, and possibly endothelial NO synthase activity.38,39 Furthermore, as lipid rafts/caveolae are membrane microdomains harboring multiple signaling platform, we cannot exclude that the effects of LXR activation may extend to other relevant receptors and pathways, such as those of fibroblast growth factor or transforming growth factor-β.

We provide evidence that the antiangiogenic effects of LXR activation can be therapeutically meaningful, because T0901317 reduced both the vascularization and tumor growth of LLC-1 grafts. Of note, the anticancer properties of T0901317 were detectable only in vivo, because this drug did not modify the proliferation and apoptosis of LLC-1 cells in vitro. The resistance of LLC-1 cells to LXR activation is in line with previous observations that LXR agonists can impair cell cycle progression and favor apoptosis (eg, by increasing the expression of p27/Kip-1) only in certain cancer cell lines, whereas others are resistant to LXR activation.5,12,13,40 It has been suggested that the sensitivity to LXR agonists may relate to the cell-specific effects of LXR agonists on cholesterol homeostasis.31 Our findings support this hypothesis, because in LLC-1 cells T0901317 did not modify the expression of both cholesterol-handling proteins.
(ABCA1, ABCG1, and low-density lipoprotein receptor) and cell cycle regulator p27/Kip-1.

Taken together, the present findings picture a scenario where LXR agonists can limit cancer growth in vivo by exerting substantial antiangiogenic effects. Nonetheless, it can be foreseen that in certain cancer types, the angiostatic properties of LXR-activating compounds may synergize with direct antiproliferative and proapoptotic actions of LXRs on tumor cells. As the systemic administration of LXR-activating drugs is presently limited by side effects, such as liver steatosis and increased low-density lipoprotein-cholesterol, these drugs may find earlier applications as locally delivered therapies.

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**References**


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SUPPLEMENT MATERIAL

Liver X Receptor activation reduces angiogenesis by impairing lipid raft localization and signaling of VEGF receptor-2

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SUPPLEMENTAL METHODS

Materials and cell culture
T0901317, GW3965 and water soluble cholesterol were purchased from Sigma, VEGF-A and FGF-2 from R&D. Pooled human umbilical vein endothelial cells (HUVEC) were cultured at 37°C in a 5% CO₂ incubator using EBM-2 medium (Lonza) supplemented with 2% fetal bovine serum (FBS), endothelial growth factors and supplements (EGM-2 medium, Lonza). For experimental treatments, HUVEC (passage 3-5) were grown to 70% confluence, incubated for 6 hours in EGM-2 containing 0.5% FBS and subsequently treated for 18 hours with the indicated compounds in the same medium, as previously described.¹ Lewis lung carcinoma cells (LLC-1 or LL/2) are a mouse (C57BL) lung cancer cell line.² LLC-1 cells were cultured at 37°C in a 5% CO₂ incubator in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% FBS.

Gene expression analysis
Total RNA was extracted from cells using affinity columns (Qiagen) and from tissues using TRIzol reagent (Invitrogen). RNA quality was checked by spectrophotometry or with an Agilent Bioanalyzer. For gene expression screening, HG-U133 Plus 2.0 microarrays (Affymetrix) were used. For each experimental condition, 2-4 biological replicates were included. Data analysis was performed with dChip Software and included normalization, computation of model-based expression indexes, filtering and
Transcripts with a fold change >2.0 or <-2.0 were considered as differentially expressed. Quantitative reverse transcription real time-PCR (qRT-PCR) was performed in a 2-step fashion using TaqMan chemistry (Sigma, Roche) on an ABI 7500 instrument (Applied Biosystems), following standard protocols. 18S rRNA was used as housekeeping gene. Gene expression levels were analyzed using the $2^{-\Delta\Delta CT}$ relative quantification system.

**Gene silencing**

High-titer lentiviral particles carrying shRNA MISSION RNA interference vectors against LXRβ (Target Set NM_007121) or scramble non-target shRNA were obtained from Sigma. The sequences of shRNAs targeting human LXRβ were as follows.

- shRNA-LXRβ_1:
  CCGGAGAGTGTATCCCTTCTGAAACTCGAGTTCAAGAAGGTGATACACTCTTTTTTT

- shRNA-LXRβ_2:
  CCGGGAAGGCATCCACTATCGAGATCTCGAGATCTCGATAGTGGATGCCCTTCTTTTT

- shRNA-LXRβ_3:
  CCGGGAAAGGCATCCACTATCGAGATCTCGAGATCTCGATAGTGGATGCCCTTCTTTTT

HUVECs were infected overnight at a multiplicity of infection (MOI) of 3, and then positively selected with 2.5 µg/ml puromycin. Down-modulation of the target protein was verified 72 hours post infection by western blot analysis.

**Cell cholesterol**

HUVEC cholesterol content was determined using a colorimetric assay (Biovision). Lipids were extracted from $2 \times 10^6$ cells using chloroform:isopropanol:NP-40 (7:11:0.1). After homogenization and spinning, the organic phase was dried and suspended in a reaction mix containing cholesterol oxidase and a colorimetric probe. Absorbance at 570 nm was finally measured in a microplate reader. The relative amount of esterified and non-esterified cholesterol was determined by incubation of the lipid extract in
the presence or absence of cholesterol esterase. In HUVEC, non-esterified cholesterol represented >95% of total cholesterol.

Cell fractionation

Light membrane fractions were isolated as described previously. Briefly, HUVECs were scraped in 0.5 M sodium carbonate buffer containing protease inhibitor cocktail (Sigma), homogenized and sonicated. Samples were adjusted to 45% sucrose by the addition of 90% sucrose prepared in MBS (25 mM Mes, pH 6.5, 0.15 M NaCl) and placed into ultracentrifugation tubes. A 5-35% sucrose discontinuous gradient was formed above, and then samples were centrifuged at 45x10³ rpm for 16 hours at 4°C in a SW-55Ti rotor (Beckman). Ten 500 µl fractions were collected from the top of each gradient and then analyzed by SDS-PAGE. For immunoblotting, equal volumes of the ten fractions were loaded in SDS-polyacrylamide gels.

Immunoblotting

Total protein extracts were obtained by boiling samples in extraction buffer (0.125 M Tris-HCl, pH 6.8, 4% SDS, 20% glycerol). Nuclear extracts were obtained with NE-PER extraction reagent (Pierce) following the manufacturer’s instruction. For immunoblotting, 20-40 µg of protein extract per sample were separated on a SDS-polyacrylamide gel and subsequently blotted to a PVDF membrane. The membrane was washed with TBS, air dried and incubated overnight with primary antibodies. After washing, the membrane was incubated with a secondary HRP-conjugated antibody and immunoreactive proteins were visualized by enhanced chemiluminescence (ECL) system (Biorad). Antibodies were obtained for Cell Signaling (P-VEGFR-2 Y1175, VEGFR-2, P-PLCγ, p27), R&D (LXRβ), Novus (ABCA1, ABCG1, CETP), Cayman (LDLR), BD (flotillin-2), Santa Cruz (vinculin, laminB, cyclin A, cyclin D1, caveolin-1, BIP, SREBP-1, γ-tubulin), VEGF (Peprotech).

Cell migration and tube formation assays
Chemotaxis assay was performed in a Boyden’s chamber using a gelatin-coated polycarbonate membrane (8 µm pore size, Neuroprobe). The lower compartment of the chamber was filled with EBM-2 medium added with VEGF-A (10 ng/mL). HUVECs were serum starved overnight in the presence of the indicated compounds, then suspended in serum free EBM-2 at a concentration of 1x10^6 cells/ml and 50 µl of this suspension was added to the upper compartment of the chamber. After 5 h of incubation at 37°C, 5% CO₂, the upper surface of the filter was scraped, and the membrane was fixed and stained with Diff-Quick (Medion Diagnostic). Three random fields for each sample were counted with a microscope. For haptotaxis assay, endothelial cells were serum starved and 5x10^4 cells were seeded in serum-free EBM2 on the upper compartment of a 8 µm pore transwell (Falcon) coated on the lower side with fibronectin (10 µg/ml, Sigma). The lower compartment was filled with serum-free EBM2. After being incubated for 5 hours at 37 °C, 5% CO₂, the upper side of the insert was scraped, cells were fixed in 2.5% glutaraldehyde for 30 min and stained with 0.1% crystal violet. Migrated cells were counted with a microscope.

For in vitro tubulogenesis assay, 130 µl of basement membrane matrix (Matrigel, BD Biosciences) were layered in a 48 wells plate and allowed to solidify. After incubation with the indicated compounds, 18x10^3 HUVEC were seeded per well and overlaid with EGM-2 containing the indicated compounds. After 8 hours, tubular structures were examined with an inverted-phase contrast microscope (Leica) and photographed for subsequent digital processing.

**Cell proliferation, cell cycle, apoptosis and cell size evaluation.**

Cell viability was determined by incubating HUVEC with MTT (0.5 mg/mL, Roche) for 4 hours in growth medium. Cells were subsequently washed with PBS and formazan crystals were solubilized. Finally, absorbance at 550-600 nm was recorded in a microplate reader. HUVEC or LLC1 proliferation rate and cell cycle were evaluated with the Click-iT EdU flow cytometry assay kit (Invitrogen) and propidium iodide (PI) staining as described. Briefly, after incubation with EdU (20 µM) for 2 hours, HUVEC or LLC1 were suspended and processed for EdU detection following the manufacturer’s instructions. Cell DNA was labeled by incubation with PI (50 µg/mL) and Ribonuclease A (100 µg/mL)
for 3 hours. Data were acquired with a CyAn ADP flow cytometer (Dako) and analyzed with FlowJo software (Tree Star). For apoptosis, annexin-positive HUVEC or LLC1 were detected using Annexin V-PE apoptosis kit (Merck). Briefly, cells were suspended in annexin buffer, incubated with annexin V-PE (1:100) for 5 minutes and finally subjected to flow cytometry. In order to evaluate cell volume changes, HUVEC were treated as described and then analyzed by flow cytometry.

**Immunofluorescence and image analysis**

HUVEC were fixed with phosphate-buffered saline 4% PFA, incubated overnight at 4 °C with anti-caveolin-1 (Santa Cruz) and anti-VEGFR2 (clone 89109, R&D systems) antibodies, followed by 1 hour incubation with secondary antibodies (Molecular Probes); nuclei were counterstained with DAPI (Molecular Probes). Images were captured with a Leica AF6000 workstation equipped with a TIRF module and analyzed with ImageJ software. Tumor sections were incubated for 1 hour at 37 °C with anti-ABCA1 (Novus Biological) and anti-CD31 (BD Biosciences) antibodies. Images were acquired by using a Leica TCS SP2 AOBS confocal microscope and analyzed with Leica Confocal Software.

**Animal studies**

Wild-type mice were 8-12 week-old female C57BL/6. The generation of LXRαβ−/− mice has been previously described. Mice were hosted under standard 12 hour light/dark cycle and fed standard rodent chow. For angioreactor implantation, mice were anaesthetized with ketamine/xylazine. In vivo experimental procedures were approved by the local Ethical Committee.

**Aortic ring angiogenic assay**

The mouse aortic ring assay was performed as previously described. In brief, thoracic aortas were removed from 8-12 week-old wild-type mice and fibro-adipose tissue was dissected away. Aortas were sectioned in 1 mm-long aortic rings and incubated for 2 days in serum-free medium with treatment compounds. 48-well culture dishes were coated with 100 µl of BME (BD Biosciences) and allowed to
solidify. Treatment compounds were added to BME before its solidification. Rings were sealed in place with an overlay of 100 µl of BME and covered with M199-10% FCS plus VEGF-A (30 ng/mL), FGF-2 (15 ng/mL) and the indicated treatment drugs. Medium was changed every 2-3 days. Tubular structures were examined with an inverted-phase contrast microscope (Leica) and photographed for subsequent digital processing.

**In vivo neoangiogenesis**

In vivo neoangiogenesis was performed using a modified Directed In Vivo Angiogenesis Assay (DIVAA, Trevigen). Briefly, one-end open silicone cylinders (angioreactors) filled with 20 µl of basement membrane extract premixed with VEGF-A (10 ng) and FGF-2 (30 ng) plus T0901317 or DMSO were implanted subcutaneously into the dorsal flank of anaesthetized mice (4 angioreactors/mouse). On day 14, mice were euthanized and angioreactors were carefully dissected and photographed. The vascularized basement membrane extract of each angioreactor was then recovered and digested using CellSperse solution (Trevigen). Vessel-derived cells were then pelleted and suspended in 500 µl PBS. Red blood cells were finally counted by FACS analysis.

**Tumor grafts**

For LLC-1 tumor grafts, 10⁶ cells were suspended in 150 µl PBS and injected subcutaneously into the back of 8-12 week-old female C57BL/6 mice. After one week, mice were randomized into a treatment group (receiving T0901317 suspended in 0.5% carboxymethyl cellulose - 0.25% Tween 20, 20 mg/kg i.p. q.d.) and a control group (receiving the same amount of vehicle). Tumor growth was checked daily by caliper measurement and tumor volume was estimated as \( V = \text{width}^2 \times \text{length}/2 \). Mice were euthanized after one week of treatment and tumors were recovered by surgical dissection. For CD31 staining, OCT-frozen samples were cut into 10 µm-thick sections. For immunostaining, sections were dried, fixed in 50:50 methanol-acetone, blocked with FBS and incubated with a primary PE-labeled anti-CD31 rat primary
antibody (BD Biosciences). Nuclear counterstaining was performed with DAPI. Microscopy and digital photography was performed with an inverted Leica microscope.

Data analysis
Quantification of tubular structures (HUVEC tubulogenesis, aortic ring assay) was performed with WinRhizo Pro software (Regent Instruments) as previously described. Quantification of CD31 staining on tumor sections was performed with ImageJ software (NIH). Data are presented as average ± standard error of the mean (SEM). Statistical significance was tested with unpaired Student’s t-test or one-way ANOVA with post-hoc analysis. The number of replicate experiments and the level of statistical significance is indicated in the figure legends.

REFERENCES FOR SUPPLEMENTAL METHODS


**SUPPLEMENTAL FIGURE LEGENDS**

**Supplementary Figure I.** (A) Microarray expression profile of genes involved in cholesterol metabolism upon treatment for 18 hours with T0901317 (T090, 1 µM) or vehicle (DMSO). Only differentially expressed genes are shown, as defined by fold-change of expression >2.0 or <-2.0 compared to DMSO. Expression levels are normalized and represented with a color scale. SD: standard deviation of expression levels. (n=4 for DMSO and n=2 for T090) (B) Relative mRNA expression (arbitrary units) of LXRβ in HUVEC infected with scramble shRNA or different LXRβ-targeting shRNAs. HUVEC were treated as in A. *p<0.05, **p<0.01 versus DMSO, n=3. (C) Nuclear levels of LXRβ protein in HUVEC infected with scramble shRNA or with different LXRβ-targeting shRNAs scramble shRNA. Representative blot of 3 experiments.
Supplementary Figure II. Relative mRNA expression (arbitrary units) of (A) ABCA1, (B) ABCG1, (C) IDOL, (D) CETP, (E) SREBP1c in HUVEC infected with scramble shRNA or with different LXRβ-targeting shRNAs. HUVEC were treated for 18 hours with T0901317 (T090, 1 µM) or vehicle (DMSO). *p<0.05, **p<0.01, ***p<0.001 versus DMSO; †p<0.05, ††p<0.01, †††p<0.001 versus T0901317-treated scramble shRNA; n=3.

Supplementary Figure III. (A) Number of forks per digital image formed by HUVEC in experiments of Matrigel tubulogenesis (see regular Figure 2C). HUVEC were treated for 18 hours with the indicated compounds: vehicle (DMSO), cholesterol (CHOL, 20 µM), GW3965 (10 µM), T0901317 (T090, 10 µM). *p<0.05 versus DMSO, †p<0.05 versus T090+CHOL, n=3. (B) Contrast-phase images of endothelial tubules formed by HUVEC treated for 18 hours with vehicle (DMSO) or with the natural LXR agonist 22-hydroxycholesterol (22OHC, 10 µM). Representative microphotograph of n=3 experiments. (C) Cell growth of HUVEC treated for 48 hours with vehicle (DMSO) or 22-hydroxycholesterol (1, 2, 5, 10 µM). Cell growth was assessed by MTT. *p<0.05, ***p<0.001 versus DMSO, n=3.

Supplementary Figure IV. (A) Relative mRNA expression (arbitrary units) of VEGF-A in HUVEC treated for 18 hours with vehicle (DMSO) or T0901317 (T090, 10 µM). *p<0.05 versus DMSO, n=3. (B) Protein levels of VEGF-A in HUVEC treated as in A. Representative blot of 3 experiments. (C) Relative mRNA expression (arbitrary units) of VEGFR2 in HUVEC treated as in A. (D) VEGF-A-induced migration of HUVEC treated as in A. *p<0.05 versus DMSO, †p<0.05 versus T090+CHOL, n=4. (E) Fibronectin-dependent haptotaxis of HUVEC treated as in A. n=6.

Supplementary Figure V. (A) Cell growth (MTT assay) of LLC-1 cells treated for 48 hours with vehicle (DMSO) or T0901317 10 µM (T090). n=10. (B) Proliferation rate of LLC-1 incubated for 24 hours in serum-free DMEM (starve), DMEM + FBS 10% + vehicle (FBS+DMSO) and DMEM + FBS 10% + T0901317 10 µM (FBS+T090). DNA incorporation of the thymidine fluorescent analogue EdU was
detected by flow cytometry. The % of proliferating cells is indicated (representative experiment, n=3). (C) Protein levels of p27/Kip-1 in LLC-1 cells treated for 24 hours with vehicle (DMSO) or T0901317 10 µM (T090). Representative blot of 3 experiments. (D) Apoptosis of LLC-1 (% of annexin-positive cells) incubated for 24 hours with DMEM without serum (starve), DMEM-FBS 10% + vehicle (FBS+DMSO), and DMEM-FBS 10% + T0901317 10 µM (FBS+T090). *p<0.05 versus starve, n=3. (E) Relative mRNA expression (arbitrary units) of LXR target gene ABCA1 in LLC-1 tumor grafts after one week of treatment with vehicle (CMC-Tween 20) or T0901317 20 mg/kg i.p. q.d. (T090). *p<0.05, n=10 mice per treatment group.
A

standardized
gene expression

-1.5 SD

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B

LXRβ

mRNA expression (A.U.)

Av Ct

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C

Supplementary Figure I
Supplementary Figure II
Supplementary Figure III
Supplementary Figure IV

A  VEGF-A
Av Ct 22.6 20.6

mRNA expression (A.U.)

T090 +

DMSO -

B  VEGFR-2
Av Ct 22.7 22.6

mRNA expression (A.U.)

T090 +

DMSO -

C  VEGF-A

165

121

Tubulin

T090 +

DMSO -

D  Chemotaxis (%)

50 75 100 125 150

DMSO  CHOL  T090  T090+CHOL

E  % haptotaxis (fibronectin)

50 100 150

DMSO  CHOL  T090  T090+CHOL
Supplementary Figure V

A  

B  

C  

D  

E  

**LLC-1**  

**T090**  

**DMSO**  

**proliferation (%)**  

**expression (A.U.)**  

**T090**  

**CMC**  

**ABCA1 mRNA**  

**Tumor**  

**Av Ct**  

**p27/Kip-1**  

**vinculin**  

**proliferation (%)**  

**FBS+DMSO**  

**FBS+T090**  

**starve**  

**SSC**  

**EdU**  

**annexin+ cells (%)**  

**EdU**  

**ABC1 mRNA expression (A.U.)**  

**C**  

**LLC-1**  

**T090**  

**DMSO**  

**p27/Kip-1**  

**vinculin**  

**LLC-1**  

**starve**  

**SSC**  

**EdU**  

**annexin+ cells (%)**  

**EdU**  

**ABC1 mRNA expression (A.U.)**

Supplementary Figure V