Telmisartan Exerts Pleiotropic Effects in Endothelial Cells and Promotes Endothelial Cell Quiescence and Survival

Mauro Siragusa, William C. Sessa

Objective—Telmisartan, an angiotensin II type 1 receptor blocker, and amlodipine, a calcium channel blocker, are antihypertensive agents clinically used as monotherapy or in combination. They exert beneficial cardiovascular effects independently of blood pressure lowering and classic mechanisms of action. In this study, we investigate molecular mechanisms responsible for the off-target effects of telmisartan and telmisartan–amlodipine in endothelial cells (ECs), using an unbiased genomic approach.

Approach and Results—In human umbilical vein ECs, microarray analysis of gene expression followed by pathway enrichment analysis and quantitative polymerase chain reaction validation revealed that telmisartan modulates the expression of key genes responsible for cell cycle progression and apoptosis. Amlodipine’s effect was similar to control.

Conclusions—Telmisartan exerts antiproliferative and antiapoptotic effects in ECs. This may account for the improved endothelial dysfunction observed in the clinical setting.

Key Words: Akt protein, human endothelial cells proliferation telmisartan
channels are not expressed or play only minor roles, such as endothelial cells (ECs). In clinical trials, AML was shown to reduce ischemia in patients with coronary artery disease and hospitalizations for unstable angina and revascularization, as well as the need for revascularization in patients with stable angina and to slow down the progression of existing atherosclerotic lesions and the onset of new ones. As for TLM, these effects are independent of BP lowering or calcium channel blockade and a link to NO biology has been suggested. Indeed, AML was shown to induce NO release and to reduce oxidative stress and inflammation, thus improving endothelial function. To explain this unexpected ability, it has been shown that AML alters caveolae integrity, increases caveolin-free endothelial NO synthase, leading to potentiated NO production in response to agonists.

Clinical evidence demonstrated that the TLM–AML combination generates a dose-dependent BP-lowering effect significantly greater than that of either agent administered as monotherapy. Such a combination is likely to provide also a better cardiovascular protection compared with monotherapies, and clinical trials are currently corroborating this hypothesis. Improvement of endothelial dysfunction by therapy with TLM alone or TLM–AML combination represents an attractive strategy to reduce onset and progression of cardiovascular disease. However, an exhaustive understanding of the molecular mechanisms underlying the beneficial effects of these drugs on the endothelium is missing. Therefore, the aim of the present study was to provide a comprehensive analysis of the potential pleiotropic actions of these agents on ECs using an unbiased genomic approach. Here, we show that TLM alone induces a state of EC quiescence by regulating networks of genes influencing cell growth and apoptosis.

Materials and Methods
Materials and Methods are available in the online-only Supplement.

Results
Telmisartan Modulates Endothelial Gene Expression
To assess the effects of TLM, AML, and TLM+AML on endothelial gene expression in an unbiased manner, we used an Affymetrix human whole transcript microarray which includes 30,000 genes. Total RNA samples were obtained from primary cultures of human umbilical vein ECs (HUVECs).
treated with dimethyl sulfoxide, TLM, or TLM+AML for 24 hours. TLM significantly modulated the expression of ≈1700 genes, whereas TLM and AML together significantly modulated ≈1500 genes (http://www.ncbi.nlm.nih.gov/geo, accession number GSE42808). Interestingly, MetaCore pathway enrichment analysis revealed that several cell cycle and proapoptotic genes were downregulated by TLM alone or in combination with AML as compared with vehicle dimethyl sulfoxide (Table).

Telmisartan Prevents the Cyclic Expression of Cell Cycle–Related Genes, Thus Inhibiting EC Proliferation

On the basis of functional relevance of genes regulating cell cycle progression, we evaluated the differential expression of selected genes by real-time polymerase chain reaction over a time course of 72 hours. Notably, TLM prevented the typical cyclic expression of Cyclin A1 (CCNA1), Cyclin B2 (CCNB2), Opa interacting protein 5 (OIP5), Aurora-A (AURKA), CDC28 protein kinase regulatory subunit 2 (CKS2), and Histone cluster 1, H2bk (HIST1H2BK) at multiple time points (Figure 1A–1F). AML alone had no effect compared with vehicle and did not influence the actions of TLM when used in combination. As a result of TLM reducing the expression of cell cycle–related genes, serum and growth factor–induced cell growth was inhibited by TLM (Figure 2A, i) and HUVECs failed to enter the S-phase of the cell cycle as shown by the prominent decrease in BrdU incorporation at 48 hours after stimulation (Figure 2A, ii). As expected, AML alone did not affect cell growth or BrdU incorporation, consistent with the idea that the above effects on gene expression are solely TLM dependent, thus prompting us to focus only on this agent. To test the dose-dependent effect of TLM on cell proliferation, HUVECs were treated with TLM at various concentrations from 0.1 to 100 µmol/L. TLM inhibited cell growth at a starting concentration of 10 µmol/L, whereas lower concentrations had no effect (Figure 2B, i). Accordingly, BrdU incorporation measured at 48 hours after treatment was significantly decreased already at 10 µmol/L, with no significant effect at lower concentrations (Figure 2B, ii). Interestingly,
TLM inhibited also the serum and growth factor–induced cell growth of human umbilical artery ECs in a dose-dependent manner, thus suggesting that its antiproliferative action is not cell type specific (Figure 2C, i and ii).

**TLM Inhibits Cell Cycle Progression Independently of AT1 Receptor Blockade**

Although all experiments were performed in the absence of exogenously added angiotensin II, we asked whether the observed antiproliferative effect could be a result of AT1 receptor blockade by TLM. We, therefore, exposed HUVECs to 2 other ARBs, losartan and valsartan, using concentrations similar to those used in the experiments involving TLM. Although losartan did not influence cell proliferation even at the highest concentration tested, valsartan seemed to induce a delay in cell growth at a concentration of 100 µmol/L (Figure 3A, i and ii). However, BrdU incorporation at 48 hours after treatment with 100 µmol/L valsartan did not differ from vehicle, suggesting that the difference in cell number was because of cell toxicity rather than an actual effect on cell cycle (Figure 3A, iii). In addition, we treated fibroblast-like COS-7 cells, which do not express AT1 receptor, with 100 µmol/L TLM and evaluated serum-induced cell growth. Similar to HUVECs and human umbilical artery EC, TLM treatment resulted in a marked inhibition of cell proliferation and S-phase entry, as documented by significantly reduced BrdU incorporation at 72 hours after stimulation (Figure 3B, i and ii).

**Telmisartan Negatively Modulates the Akt Signaling Pathway**

To uncover the molecular mechanisms underlying the antiproliferative effect of TLM, we performed a bioinformatic analysis of the cell cycle–related genes modulated by TLM using GeneWeb analysis of gene interactions (SABioscience platform). The expression of some genes (eg, CCNA1, AURKA, and CKS2) is reportedly repressed by p53, whereas others (eg, HIST1H2BK) are under the positive transcriptional control of E2F. Both p53 and E2F stability and thus activity can be regulated by the Akt pathway via MDM2 and CyclinD1, respectively. It is, indeed, well established that the Akt pathway regulates proliferation and cell survival. We, therefore, investigated the activation of Akt and downstream signaling over a time course spanning from 10 minutes to 72 hours after serum stimulation. Western blot analyses showed that TLM treatment significantly inhibited the full activation of Akt by reducing phosphorylation at the mammalian target of rapamycin complex 2–dependent site S473 and the 3-phosphoinositide-dependent kinase 1–dependent site, T308, throughout the time course (Figure 4A and quantified in Figure 4B, i and ii). Interestingly, Akt-dependent phosphorylation of MDM2 at the S166 residue was also decreased, particularly at the later time points, rendering the ubiquitin ligase less active (Figure 4A and quantified in Figure 4B, iii). MDM2 mediates p53 ubiquitination and degradation. We, therefore, hypothesized that because MDM2 was less active, p53 would accumulate in the nucleus and act as a repressor for genes important for cell cycle progression. Consistent with this hypothesis, immunofluorescence and Western blot analyses showed that TLM-treated HUVECs retained higher amounts of p53 in the nucleus as compared with control, starting at 24 hours until 72 hours after serum stimulation (Figure 4C, i and iii and quantified in ii and iv). In addition, Akt-dependent phosphorylation of glycogen synthase kinase-3β (S9 residue) was assessed. As expected, TLM induced a decrease of glycogen synthase kinase-3β phosphorylation, resulting in higher kinase activity (Figure 4A and quantified in Figure 4B, iv). Glycogen synthase kinase-3β is known to
Telmisartan Protects ECs From Serum Starvation–Induced and 7-Ketocholesterol–Induced Apoptosis

TLM treatment resulted in the downregulation of voltage-dependent anion channel 1 (VDAC1), voltage-dependent anion channel 2 (VDAC2), solute carrier family 25 (mitochondrial carrier; adenine nucleotide translocator), member 4 (SLC25A4), and direct inhibitor of apoptosis–binding protein with low pI (DIABLO), involved in the induction of apoptosis via the mitochondrial pathway and in the upregulation of BCL2-like 2 (BCL2L2), which reportedly promotes cell survival.27 First, we validated the microarray results by real-time polymerase chain reaction (Figure 5A and quantified in Figure 5B, i and ii). Moreover, serum-starved cells undergoing apoptosis in the control group exhibited characteristic apoptotic bodies formation and stained positively for annexin V, whereas TLM-treated cells did not (Figure 5B, ii and iii). Oxysterols constitute the major toxic component in oxidized low-density lipoprotein and are present in human atheromatous lesions.28 Oxysterols, such as 7-ketocholesterol, contribute to endothelial dysfunction and induce EC apoptosis via the mitochondrial pathway.29,30 Accordingly, control HUVECs treated with 7-ketocholesterol showed a significant increase in caspase-3 activity, whereas TLM exerted a protective effect (Figure 5D).

Discussion

Although an increasing numbers of clinical and experimental results indicate the effectiveness of TLM and AML in cardiovascular protection, independently of their BP-lowering action, little is known about the molecular mechanisms responsible for the observed pleiotropic effects. Given the importance of the endothelium in the maintenance of vascular homeostasis, in the current study, we investigated the effects of TLM and TLM+AML combination on a well-defined model of endothelium using an unbiased genomic approach.

In the absence of angiotensin II stimulation, TLM alone or in combination with AML significantly blunted the expression of several genes, whose encoded products are involved in the regulation of the cell cycle. Among these, CyclinA1 and CyclinB2 are involved in the control of the cell cycle at the G1/S (start) and G2/M (mitosis) transitions, respectively.31,32 Knockdown of OIP5 expression has been reported to inhibit cell growth of several cancer cell lines.33,34 The serine/threonine kinase Aurora-A plays a pivotal role in several mitotic events, including the establishment of mitotic spindle, centrosome duplication, centrosome separation, as well as maturation, chromosomal alignment, spindle assembly checkpoint, and cytokinesis.35 CKS2 associates with cyclin-dependent kinases and has been shown to play a direct role in cell cycle regulation. Its knockdown causes cessation of proliferation and cell cycle arrest because of impaired transcription of key genes involved in cell cycle progression.36 Finally, Histone cluster 1, H2bk, is a core component of the nucleosome and influences the compaction of chromatin into higher order structures. Our results revealed that the contribution of AML to the overall downregulation of the cell cycle machinery is negligible, whereas TLM is solely accountable for inducing a state of EC quiescence. As expected from our gene expression studies, we found that TLM inhibited cell proliferation in HUVECs, as well as human umbilical artery EC in a dose-dependent manner beginning at a concentration of 10 µmol/L, whereas AML had no effect. Moreover, COS-7 cells, which lack the AT1 receptor, displayed cell growth impairment when treated with TLM, underscoring that the antiproliferative effect of TLM relies neither on AT1 receptor blockade nor on the cell type used. This finding is in line with previous studies demonstrating a dose-dependent antiproliferative action of TLM on human vascular smooth muscle cells, cardiac fibroblasts, and murine macrophages, by activating PPARγ-dependent and independent pathways.8,37,38 Interestingly, our

Figure 3. Telmisartan (TLM)-induced inhibition of cell proliferation is independent of angiotensin II type 1 receptor blockade. A, Dose-dependent effects of losartan (LOS) or valsartan (VAL) on cell growth (i and ii, respectively) and BrdU incorporation (iii) in human umbilical artery endothelial cells (HUVECs). B, Growth curve (i) and BrdU incorporation assay (ii) in COS-7 cells treated with 100 µmol/L TLM for the indicated times (n=3); *P<0.05 vs dimethyl sulfoxide (DMSO).
gene expression analysis did not highlight any significant changes in the expression of neither PPARγ itself nor genes downstream of PPARγ, suggesting that TLM influences EC physiology via PPARγ-independent mechanisms. In addition, our data support the notion that TLM activation of PPARγ is cell type specific.

Figure 4. Negative modulation of Akt signaling accounts for antiproliferative action of telmisartan (TLM). Immunoblot analyses (A) and averaged densitometric data (B) show the effects of 100 µmol/L TLM on the dynamic phosphorylation of Akt (i and ii), MDM2 (iii), glycogen synthase kinase (GSK)-3β (iv) and on CyclinD1 levels (v) over a time course of 72 h in human umbilical artery endothelial cells (HUVECs). C, Representative immunofluorescence images (i), immunoblot analyses (iii) and respective averaged fluorescence intensity quantification (ii) and averaged densitometric data (iv) of nuclear p53 in HUVECs treated as in A (scale bar, 50 µm; n=5); for all data in B and C, P<0.05 vs dimethyl sulfoxide (DMSO) by 2-way ANOVA for both the effect of time and the treatment on fold change. FBS indicates fetal bovine serum.
In this study, we interrogated potential mechanisms responsible for the antiproliferative effects of TLM in ECs by investigating its impact on Akt activation and downstream signaling. In line with another study showing that TLM inhibits vascular smooth muscle cell proliferation by inhibiting Akt activation,37 we found that TLM attenuates Akt phosphorylation and activation, ultimately resulting in accumulation of p53 in the nucleus, where it represses the transcription of genes necessary for cell cycle progression. In addition, TLM-treated ECs displayed lower amounts of CyclinD1 as compared with control, which suggest diminished activation of E2F-dependent G1/S-phase gene expression. Taken together, we demonstrate a novel mechanism of action of TLM in ECs, beyond its ability to block AT1 receptor or activate PPARγ. How TLM interferes with Akt activation remains speculative, although the time course of our experiments would point to interference with the initial steps of the pathway (ie, diminished generation of phosphoinositides through reduction of phosphatidylinositol 3-kinase activity or enhancement of phosphatase and tensin homolog activity, or perhaps via direct inhibition of mammalian target of rapamycin complex 2 or 3-phosphoinositide-dependent kinase 1).

Despite the negative modulation of the Akt signaling pathway, TLM treatment did not result in elevated baseline cell death as compared with control. On the contrary, TLM protected ECs from apoptosis induced by serum/growth factors withdrawal or 7-ketocholesterol treatment. Given that the Akt pathway is not completely abrogated by TLM, and the extent of its activation seems to be enough to support cell survival, the antiapoptotic effect seems to be because of a direct downregulation of key components of the apoptotic machinery in mitochondria (ie, the channels participating in the formation of the permeability transition pore complex, responsible for the release of cytochrome c into the cytosol, leading to activation of caspases: adenine nucleotide translocator, ANT [encoded by the gene SLC25A4], VCAD1, and VCAD2).39,40 Second mitochondria-derived activator of caspases (Smac)/Diablo, another mitochondrial gene whose expression is downregulated by TLM, promotes apoptosis by binding and blocking the activity of inhibitor of apoptosis protein, a negative regulator of apoptosis.41 However, TLM promotes EC survival by upregulating the BCL2L2 gene expression, which encodes the antiapoptotic Bcl-2 family member Bcl-W.27

Given the higher proliferation rate of the endothelium in atherosclerotic susceptible regions42 and the role that endothelial proliferation and apoptosis play in the stability of the...
atherosclerotic plaque, 43,44 the finding that TLM promotes EC quiescence and survival, together with its known anti-inflammatory and antioxidative effect on the endothelium, may have important implications for the antiatherogenic and plaque-stabilizing actions of this agent.

In line with most of the studies in the literature comparing the effects of TLM with those of other ARBs in multiple cell types, 8,11,37,38 2 other ARBs tested in our study, losartan and valsartan, failed to inhibit EC growth, underscoring the fact that TLM holds unique anti-proliferative properties, not shared by other drugs belonging to the same family and that this effect is entirely independent of AT1 receptor blockade. Surprisingly, despite evidence that AML directly regulates EC functions, 13,17 we could not identify any major effect of this agent on isolated ECs, implying that AML may modify posttranslational processes that do not result in quantitative changes in gene expression.

In summary, by analyzing global changes in EC gene expression, we have shed light into novel mechanisms by which TLM may help prevent endothelial dysfunction, thus protecting against the development and progression of cardiovascular disease in patients with hypertension.

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Disclosures

None.

References


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Pleiotropic Effects of Telmisartan in Endothelial Cells


Significance

Telmisartan, an angiotensin II type 1 receptor blocker, is a clinically used antihypertensive agent, which exerts beneficial cardiovascular effects independently of blood pressure lowering and classic mechanism of action. This is the first study investigating the molecular mechanisms responsible for the pleiotropic actions of telmisartan on primary endothelial cells, using a genome-wide approach. We show that telmisartan negatively modulates the expression of key genes involved in cell cycle progression and induces a state of endothelial cell quiescence by affecting the Akt/MDM2/p53 and Akt/glycogen synthase kinase-3β/CyclinD1 signaling pathways. Moreover, telmisartan promotes endothelial cell survival by inducing downregulation of proapoptotic genes. Thus, our data support the idea that telmisartan can uniquely protect and preserve the endothelium beyond angiotensin II type 1 receptor antagonism.
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Materials and Methods

Reagents
All reagents were purchased from Sigma-Aldrich unless otherwise specified.

Cell culture
Human umbilical vein endothelial cells (HUVEC) were obtained from the Yale University Vascular Biology and Therapeutics Core facility, plated on 0.1% gelatin coated dishes in M199 media supplemented with endothelial cell growth supplement (ECGS), 10% fetal bovine serum (FBS), penicillin-streptomycin and glutamine and used between passages 2 and 4. Human umbilical artery endothelial cells (HUAEC, Clonetics Lonza) were plated on 0.1% gelatin coated dishes in EBM-2 supplemented with EGM2-MV SingleQuots (Lonza) and 5% FBS and used between passages 6 and 8. COS-7 cells were cultured in DMEM supplemented with 10% FBS, penicillin-streptomycin and glutamine. Cultures were kept in a humidified incubator at 37°C in an atmosphere containing 5% CO₂. In all experiments, culture media were supplemented with the indicated concentrations of TLM (Sigma-Aldrich, cat. n. T8949 or Boehringer-Ingelheim, Ridgefield, CT), losartan (LOS, Cayman chemical, cat. n. 10006594), valsartan (VAL, Sigma-Aldrich, cat. n. SML0142) or AML (Sigma-Aldrich, cat. n. A5605) in dimethyl sulfoxide (DMSO) or an equivalent volume of DMSO alone, as control, for the indicated times. Media with drugs were replaced every 24 hours.

Gene expression analysis
HUVEC were cultured for 24 hours in complete media containing DMSO, 100 µmol/L TLM or both 100 µmol/L TLM and 5 µmol/L AML. Total RNA was isolated using the miRNeasy Mini kit (Qiagen) according to the manufacturer’s instructions. RNA samples were assessed with a NanoDrop 2000c spectrophotometer (Thermo Scientific) and run on a bioanalyzer to determine acceptable quality and quantity. In order to generate sense-strand cDNA, samples were processed using The Ambion® WT Expression Kit (Applied Biosystems), followed by the GeneChip® WT Terminal Labeling Kit (Affymetrix) for fragmentation and biotin labeling, according to the manufacturer’s protocol. Samples were then hybridized for 16 hours at 45°C to the Affymetrix Whole Transcript GeneChip Human Gene 1.0 ST array using the GeneChip® Hybridization, Wash, and Stain Kit (Affymetrix). Microarray analysis was carried out on two biological replicates per group. In accordance with MIAME (Minimum Information About a Micro-array Experiment) regulations, all data are available at the NCBI Gene Expression Omnibus (GEO) (http://www.ncbi.nlm.nih.gov/geo) under accession number GSE42808.

Pathway analysis and data mining of microarray data
Microarray data were filtered by signal intensity to exclude transcripts whose signal was below background level. Only transcripts with significantly different expression levels between groups were included in the pathway enrichment analysis, which was performed using the MetaCore database and software suite.
Validation of microarray data

Relevant microarray data were validated by quantitative RT-PCR. Total RNA samples collected from HUVEC treated with DMSO, 100 µmol/L TLM, 5 µmol/L AML or both TLM and AML for 12, 24, 48 or 72 hours were retro-transcribed using the RT² First Strand Kit (SABiosciences) and then assayed using Custom Human RT² Profiler PCR Arrays with proprietary wet-bench validated primers (SABiosciences), according to the manufacturer’s instructions. Alternatively, retro-transcription was done using the iScript cDNA Synthesis Kit (Bio-Rad), followed by quantitative PCR using the iQ SYBR Green Supermix (Bio-Rad) and primers designed according to published sequences: VDAC1 fwd-CGGAATAGCAGCCAAGTATCA, rev-CTGGCTTTAGAGTCTGAGTGTATC; VDAC2 fwd-GGTTCAGCTGTCTTTGGTTATG, rev-GTAGCCCACTGCAAAGTTATTC; SLC25A4 fwd-GTCTCTGTCCAAGGCAATCATTA, rev-TCACACTCTGGGCAATCATC; DIABLO fwd-CGCAGATCAGGCCTCTATAAC, rev-CGTTCCCTAAATCCCAACTCATC; BCL2L2 fwd-CTATAGGTGTGGGCACATGAAA, rev-CGTTCCCTAAATCCCAACTCATC. Validation experiments were carried out on three biological replicates and averaged.

Immunoblot analysis

HUVEC were serum starved (0.5% FBS, no ECGS) overnight. During the last 30 min before stimulation, cells were pre-incubated with DMSO or 100 µmol/L TLM, followed by stimulation with complete media containing 10% FBS and ECGS for the indicated times in the presence of DMSO or 100 µmol/L TLM. Cells were then washed with ice-cold PBS and immediately resuspended in lysis buffer (50 mmol/L Tris-HCl, 1% NP-40, 0.1% SDS, 0.1% Deoxycholic Acid, 0.1 mmol/L EDTA, 0.1 mmol/L EGTA, protease and phosphatase inhibitors) with the aid of cell scrapers, sonicated and incubated for 30-45 min on ice. Protein extracts (30-100 µg) were separated by SDS-PAGE and then transferred to 0.45 micron nitrocellulose membranes (Bio-Rad). Membranes were probed with primary antibodies against phospho-Ser473 (clone D9E, cat n.4060), phospho-Thr308 (clone L32A4, cat. n. 5106) and total Akt (cat. n. 9272), phospho-Ser166 MDM2 (cat. n. 3521), p53 (clone 7F5, cat. n. 2527), Histone H3 (clone D1H2, cat. n. 4499), phospho-Ser9 (cat. n. 9336) and total GSK3β (clone 27C10, cat. n. 9315, Cyclin D1 (clone DCS6, cat. n.2926) (all from Cell Signaling Technology) and Hsp90 (Santa Cruz Biotechnology, cat. n. 13119), followed by species specific secondary antibodies anti-IgG conjugated with either AlexaFluor 680 (Invitrogen) or IRDye800 (Rockland). Blots were washed and visualized using a LI-COR Odyssey imager. Each Immunoblot analysis was carried out on five biological replicates. Densitometric analyses were performed using the Image J software.

Immunofluorescence

HUVEC were seeded on 8-well chamber slides coated with 0.1% gelatin at a density of 5x10³ cells/well. Once adherent, cells were treated as described in the immunoblot analysis section (see above). Cells were then fixed in 4% paraformaldehyde in PBS for 15 min and permeabilized with 0.3% Triton X-100 in PBS for 30 min at RT. Cells were then incubated in 5% goat serum diluted in 0.3% Triton X-100 for 1 hour at RT, followed by incubation overnight at +4°C with a primary antibody against p53 (Cell Signaling Technology, clone 1C12, cat. n. 2524) diluted in PBS/0.3% Triton X-100. Cells were then incubated with a goat anti-mouse AlexaFluor 568 secondary antibody (Invitrogen) for 1 hour at RT, in the dark. A matched isotype control was included in each staining to
check for unspecific binding of the primary antibody. Slides were mounted with fluorescence mounting medium containing DAPI and analyzed using a ZEISS Axiovert 200M fluorescence microscope. Nuclear fluorescence intensity was measured for each cell using the ‘count density (red)’ tool in the Image-Pro Plus software and then normalized by the number of nuclei analyzed in each group.

**Functional assays on cultured cells**

**Cell growth.** HUVEC, HUAEC or COS-7 cells (4 to 15x10⁴ cells/well) were seeded in 6-well plates and serum starved (HUVEC: 0.5% FBS, no ECGS overnight; HUAEC: EBM-2 media, no FBS for 3 hours; COS-7: 0.5% FBS overnight). Cells were then stimulated with complete media containing 10% FBS and ECGS (HUVEC), EGM2-MV media containing 5% FBS (HUAEC), or 10% FBS (COS-7) for the indicated times in the presence of DMSO, various concentrations of TLM (0.1 to 100 µmol/L), 5 µmol/L AML, both TLM and AML or various concentrations of LOS or VAL (1 to 100 µmol/L). After trypsinization, the number of cells was counted with a hemocytometer. Three replicates of each condition were performed in each of three independent experiments.

**Cell proliferation.** Cells (5x10³ cells/well) were seeded in 96-well plates until adherent, serum starved and treated as described above (cell growth). In the last 20 hours before assay, cells were incubated in the presence of BrdU. BrdU incorporation was detected using the Cell Proliferation ELISA, BrdU colorimetric kit (Roche, cat. n. 11647229001) according to the manufacturer’s instructions. Five replicates of each condition were performed in each of three independent experiments.

**Apoptosis assay.** Cells were pre-incubated with DMSO or TLM for 24 hours in complete media containing 10% FBS and ECGS (HUVEC) or 5% FBS (HUAEC) and then treated with 1) growth factors- and serum-free media containing 0.1% albumin for 4 hours or 2) complete media containing 10% FBS and ECGS supplemented with either DMSO (vehicle) or 20 µg/mL 7-ketocholesterol (7-KC) for 20 hours (HUVEC only), in the presence of DMSO or TLM. The extent of apoptosis was immediately assessed by measuring Caspase-3 activity by using the Caspase-3 Colorimetric Assay kit (R&D Systems, cat. n. BF3100), according to the manufacturer’s instruction. To confirm cell death by apoptosis, HUVEC treated as described above were stained with the Alexa Fluor 488 Annexin V/Dead Cell Apoptosis Kit, following the manufacturer’s instruction, and observed using a ZEISS Axiovert 200M fluorescence microscope. Three replicates of each condition were performed in each of three independent experiments.

**Statistical analysis**

Microarray datasets were compared by one tailed unpaired t test. Results are presented as means±SEM. All differences which returned a $P \leq 0.05$ were considered significant and taken in consideration for further analysis. All experiments where the effects of two variables (e.g. time and treatment or two different treatments) were tested were analyzed by 2-way ANOVA followed by Bonferroni post hoc tests. Differences between two groups were compared by unpaired Student t test. $P \leq 0.05$ was considered significant.