Biomedicine & Pharmacotherapy 141 (2021) 111871

Contents lists available at ScienceDirect

Biomedicine & Pharmacotherapy

journal homepage: www.elsevier.com/locate/biopha

Selective estrogen receptor modulators (SERMs) affect cholesterol homeostasis through the master regulators SREBP and LXR

María E. Fernández-Suárez [a,b,*,1], Lidia Daimiel [a,b,2], Gemma Villa-Turégano [a], María Vázquez Pavón [a,3], Rebeca Busto [a,b], Joan C. Escolà-Gil [c,d,e], Frances M. Platt [f], Miguel A. Lasunción [a,b], Javier Martínez-Botas [a,b], Diego Gómez-Coronado [a,b,*]

[a] Servicio de Bioquímica-Investigación, Hospital Universitario Ramón y Cajal, IRYCIS, Ctra. de Colmenar, km 9, 28034 Madrid, Spain
[b] CIBER de Fisiopatología de la Obesidad y Nutrición (CIBEROBN), Instituto de Salud Carlos III, Spain
[c] Institut de Recerca de l'Hospital de la Santa Creu i Sant Pau, Institut d'Investigacions Biomèdiques (IIB) Sant Pau, Barcelona, Spain
[d] CIBER de Diabetes y Enfermedades Metabólicas Asociadas (CIBERDEM), Instituto de Salud Carlos III, Spain
[e] Departament de Bioquímica i Biologia Molecular, Universitat Autònoma de Barcelona, Spain
[f] Department of Pharmacology, University of Oxford, Mansfield Road, Oxford OX1 3QT, United Kingdom

ARTICLE INFO

Keywords:
Tamoxifen
Raloxifene
Toremifene
SREBP
LXR
Cholesterol

ABSTRACT

Selective estrogen receptor modulators (SERMs) are nonsteroidal drugs that display an estrogen-agonist or estrogen-antagonist effect depending on the tissue targeted. SERMs have attracted great clinical interest for the treatment of several pathologies, most notably breast cancer and osteoporosis. There is strong evidence that SERMs secondarily affect cholesterol metabolism, although the mechanism has not been fully elucidated. In this study, we analysed the effect of the SERMs tamoxifen, raloxifene, and toremifene on the expression of lipid metabolism genes by microarrays and quantitative PCR in different cell types, and ascertained the main mechanisms involved. The three SERMs increased the expression of sterol regulatory element-binding protein (SREBP) target genes, especially those targeted by SREBP-2. In consonance, SERMs increased SREBP-2 processing. These effects were associated to the interference with intracellular LDL-derived cholesterol trafficking. When the cells were exposed to LDL, but not to cholesterol/methyl-cyclodextrin complexes, the SERM-induced increases in gene expression were synergistic with those induced by lovastatin. Furthermore, the SERMs reduced the stimulation of the transcriptional activity of the liver X receptor (LXR) by exogenous cholesterol. However, their impact on the expression of the LXR canonical target *ABCA1* in the presence of LDL was cell-type dependent. These actions of SERMs were independent of estrogen receptors. We conclude that, by inhibiting the intracellular trafficking of LDL-derived cholesterol, SERMs promote the activation of SREBP-2 and prevent the activation of LXR, two master regulators of cellular cholesterol metabolism. This study highlights the impact of SERMs on lipid homeostasis regulation beyond their actions as estrogen receptor modulators.

1. Introduction

In mammalian cells, cholesterol levels are tightly regulated, mainly by two families of transcription factors, the sterol regulatory element-binding proteins (SREBP) and the liver X receptors (LXR), which govern the supply and removal, respectively, of cholesterol. The SREBP

Abbreviations: BSA, bovine serum albumin; CholMCD, cholesterol-MCD complex; DHE, dehydroergosterol; DMSO, dimethyl sulfoxide; ER, estrogen receptor; E2, 17β-estradiol; FBS, fetal bovine serum; INSIG, insulin-induced gene proteins; LDL, low-density lipoprotein; LDLR, LDL receptor; LOV, lovastatin; LPDS, lipoprotein deficient serum; LXR, liver X receptor; LXRE, LXR response element; MCD, methyl-β-cyclodextrin; PBS, phosphate-buffered saline; PMA, phorbol 12-myristate 13-acetate; RAL, raloxifene; SERM, selective estrogen receptor modulator; RXR, retinoid X receptor; SCAP, SREBP cleavage-activating protein; SREBP, sterol regulatory element-binding protein; TAM, tamoxifen; TOR, toremifene.

* Corresponding authors at: Servicio de Bioquímica-Investigación, Hospital Universitario Ramón y Cajal, IRYCIS, Ctra. de Colmenar, km 9, 28034 Madrid, Spain.
E-mail addresses: maria.fernandez-suarez@pharm.ox.ac.uk (M.E. Fernández-Suárez), diego.gomez@hrc.es (D. Gómez-Coronado).
[1] Present address: Department of Pharmacology, University of Oxford, Mansfield Road, Oxford OX1 3QT, United Kingdom.
[2] Present address: IMDEA Food, Ctra. Cantoblanco, 8, 28049 Madrid, Spain.
[3] Present address: Department of Physiology, National University of Singapore, Singapore 117597, Singapore.

https://doi.org/10.1016/j.biopha.2021.111871
Received 26 March 2021; Received in revised form 10 June 2021; Accepted 28 June 2021
Available online 3 July 2021

family of basic-helix-loop helix transcription factors is composed of three members, SREBP-1a and SREBP-1c, encoded by the same gene, *SREBF1*, but driven by different promoters and alternative splicing, and SREBP-2, encoded by a different gene, *SREBF2* [1]. SREBPs reside in the membrane of the endoplasmic reticulum, bound to SREBP cleavage-activating protein (SCAP). SCAP contains a sterol sensing domain that upon sterol biding adopts a conformation that allows it to interact with insulin-induced gene proteins (INSIG). Under these conditions, the whole complex is retained in the endoplasmic reticulum. When cholesterol levels descend, lack of sterol binding to the sterol sensing domain produces a conformational change in SCAP, leading to the disruption of the interaction with INSIG. As a result, the SREBP-SCAP complex is released from the endoplasmic reticulum and transported to the Golgi, where two proteases, S1P and S2P, sequentially cleave SREBPs yielding the amino-terminal fragment, which is the active transcription factor [2]. In the nucleus, active SREBPs bind to sterol response elements to promote the transcription of their target genes. SREBP-1c increases the expression of genes involved in fatty acid and triglyceride synthesis, SREBP-2 stimulates the expression of genes required for cholesterol biosynthesis and uptake, while SREBP-1a increases the expression of both cholesterogenic and lipogenic genes [1].

The LXR family includes two members, LXRα and LXRβ, encoded by different genes. They belong to the nuclear hormone receptor superfamily of ligand-activated transcription factors and form obligate heterodimers with the retinoid X receptors (RXRs) [3]. Natural ligands of LXR are some cholesterol-derived oxysterols, such as 22(*R*)-hydroxycholesterol and 27-hydroxycholesterol, and 24(*S*),25-epoxycholesterol, synthesized in a shunt pathway of cholesterol biosynthesis [4]. Upon cellular cholesterol overload, cholesterol-derived oxysterol levels increase in a coordinated fashion. These oxysterols bind to and activate LXR, promoting its interaction with LXR response elements (LXREs) in the regulatory regions of LXR target genes [5]. This results in the transcription of genes necessary for reverse cholesterol transport, including cholesterol efflux from cells of peripheral tissues through ATP-binding cassette (ABC) A1 and ABCG1, and cholesterol excretion from the liver through ABCG5 and ABCG8 [5]. The coordinated, tissue-specific actions of the LXR pathway maintain not only systemic cholesterol homeostasis, but also regulate immune and inflammatory responses [6].

Selective estrogen receptor modulators (SERMs) are a group of diverse, non-steroidal molecules characterised by their ability to bind the estrogen receptors (ER), ERα and ERβ [7]. They act as ER agonists or antagonists depending on the cell type and tissue targeted, although estrogen receptor-independent effects have also been described [8–10]. The SERMs tamoxifen (TAM) and toremifene (TOR), triphenylethylene derivatives differing only by the presence of a chlorine atom in the ethyl chain of TOR, are widely used to treat breast cancer. Raloxifene (RAL), another SERM, is a benzothiophene derivative indicated in Europe for the treatment of osteoporosis in postmenopausal women [11,12].

We have previously demonstrated that the SERMs TAM, RAL, and TOR increase the expression and activity of the low-density lipoprotein (LDL) receptor (LDLR) [13,14]. This effect is synergistic with that of lovastatin (LOV), a competitive inhibitor of the rate-limiting cholesterogenic enzyme 3-hydroxy-3-methylglutaryl coenzyme A reductase, in lymphoblastic MOLT-4 and hepatocytic HepG2 cell lines, but not in human primary lymphocytes [13,14]. Furthermore, we and others have shown that SERMs reduce cholesterol biosynthesis by inhibiting the activity of post-lanosterol enzymes [13,15,16]. These effects may account for the low levels of circulating cholesterol described in patients taking these drugs [15,17–19]. We have also found that SERMs block the egress of LDL-derived cholesterol from lysosomes, thus explaining the synergistic effect with LOV on LDLR expression in cell lines [13,14]. Moreover, these drugs downregulate the expression of ABCA1 and ABCG1 [14,20], resulting in reduced cholesterol efflux from macrophages [20]. The effects of SERMs described above are independent of ERs [14,20] and may contribute to the anti-tumoral efficacy of these drugs [19,21]. However, the molecular mechanisms underlying the alteration of cellular cholesterol metabolism by SERMs are not fully established. We hypothesized that the SERM-induced impairment of intracellular cholesterol trafficking leads to reciprocal alterations in the SREBP and LXR pathways. In the present work, our aim was to investigate the effect of SERMs and their combinations with LOV on the cholesterol-mediated regulation of the SREBP and LXR pathways.

2. Materials and methods

2.1. Materials

Tamoxifen citrate and raloxifene hydrochloride were obtained from Tocris Bioscience, and toremifene citrate was purchased from Sigma. Lovastatin and dimethyl sulfoxide (DMSO) were obtained from Merck Sharp & Dohme (MSD), 17β-estradiol (E2) and phorbol 12-myristate 13-acetate (PMA) from Sigma and T0901317 from Tocris Bioscience. Methyl-β-cyclodextrin was obtained from Trappsol (CTD, Inc) and 2-hydroxypropyl-β-cyclodextrin from Fluka. Other chemicals used were of analytical reagent grade quality.

RPMI 1640 culture medium, DMEM (1 mg/ml glucose) with or without phenol red were obtained from PAA Laboratories. RPMI 1640 without phenol red and fetal bovine serum (FBS) were obtained from Gibco. Dextran/charcoal treated FBS, with reduced levels of estrogens, was from HyClone. Antibiotics for supplementing the medium and non-essential amino acids were obtained from Gibco, glutamine was from Sero-Med and sodium pyruvate from Sigma. Trypsin was obtained from Sigma and phosphate-buffered saline (PBS) from Biomérieux.

2.2. Low density lipoprotein (LDL) and lipoprotein deficient serum (LPDS) preparation

Human LDL (1.019–1.063 kg/l) was isolated by vertical rotor density gradient ultracentrifugation of plasma from hypercholesterolemic patients, as previously described [22]. AcLDL was prepared from LDL by the addition of acetic anhydride [22]. LPDS was prepared from FBS or charcoal/dextran-treated FBS by ultracentrifugation at a density of 1.21 kg/l.

2.3. Preparation of cholesterol-methyl-β-cyclodextrin complexes (CholMCD)

The complex was prepared by the method previously described with minor modifications [23,24]. In brief, a solution of 14.7 mg/ml cholesterol in 100% ethanol was added in small aliquots to a stirred solution of 5% (w/v) methyl-β-cyclodextrin (MCD) on a heating block (60 °C) until a clear solution was achieved. The solution was lyophilized, and the dried complex was reconstituted in water to a sterol concentration of 3 mM. The sterol to cyclodextrin molar ratio was 1:10.

2.4. Cells and experimental design

MOLT-4 (ATCC CRL-1582) and THP-1 cells (ATCC TIB-202) were maintained in RPMI 1640 supplemented with 10% FBS, 2 mM glutamine, 100 U/ml penicillin, 100 U/ml streptomycin and 10 μg/ml gentamicin at 37 °C in humidified atmosphere with 5% CO_2. THP-1 monocytes were differentiated to macrophages with 50 ng/ml PMA for 48 h. HepG2 (ATCC HB-8065) and MCF-7 (ATCC HTB-22) cells were maintained in DMEM supplemented with 10% FBS, 0.1 mM non-essential amino acids, 1 mM sodium pyruvate, and the above-mentioned additives.

For experiments, cells were pre-treated with the corresponding medium supplemented with 10% LPDS for 24 h. Subsequently, TAM, RAL, or TOR (10 μM final concentration), LOV (1 μM), T0901317 (1 μM), the indicated amounts of E2 or vehicle (DMSO, final concentration ≤0.044%, v/v) were added to the media as indicated, and 1 h later LDL

(120 μg/ml cholesterol) or the indicated amounts of CholMCD were added to the treatments. 16 h after the addition of the drugs, E2 or vehicle, the cells were washed and processed to perform the corresponding analysis. For ER-related experiments, DMEM without phenol red and supplemented with 10% charcoal/dextran-treated LPDS was used.

2.5. DNA microarrays

The focused cDNA expression microarray (Cholestchip™) developed by our group was used in these experiments [25]. This microarray, that contained cDNA probes for 319 genes involved in lipid metabolism and cell cycle, was produced using a SpotArray 72 spotter (Perkin-Elmer) with TeleChem Stealth SMP3 split pins. The full-length cDNA probes were selected from the I.M.A.G.E. Consortium database and obtained from Open Biosystems. cDNAs were amplified using universal primers (M13 (−21) universal forward 5′-TGTAAAACGACGGCCAGT-3′ and M13/pUC reverse primer 5′-CAGGAAACAGCTATGACC-3′). Quality of the PCR products was routinely checked on agarose gel and by sequencing prior to spotting. Each PCR sample was resuspended in Micro Spoting Solution (ArrayIt) and spotted three times in two different locations on aminoxylan-treated glass slides (CapitalBio OPAminoSlide). Lucidea Universal ScoreCard probes (GE Healthcare Life Sciences) were spotted three times at the beginning and at the end of each subarray and used to validate the quality of the experiments. The microarray contains a total of 2800 spots distributed in four subarrays. DNA was fixed to the slides by irradiation of 300 mJ of UV light (GS Gene Linker, BioRad) and subsequent incubation at 80 °C for 2 h. After each series of prints, SyberGold staining and hybridisations with sense and antisense Cy5-labeled M13 were performed to analyse the quality of the print.

2.5.1. DNA Labeling and microarray hybridisation

Total RNA was extracted using Tri Reagent (Sigma) according to the manufacturer's protocol. The mRNA was isolated from 100 μg of total RNA using oligo(dT) cellulose columns from the GenElute™ mRNA MiniPrep Purification Kit (Sigma) and following the manufacturer's instructions. The mRNA was precipitated with 30:1 (v/v) 100% ethanol: 7.5 M sodium acetate, and glycogen at −20°C for 16–20 h. After precipitation, the mRNA was washed with 75% ethanol and resuspended in DEPC water. mRNA integrity was verified on agarose gel.

1 μg of mRNA was labeled with Cy3-dUTP (control) or Cy5-dUTP (test) (GE Healthcare Life Sciences) in a reverse transcription reaction with 0.5 μg/μl oligo(dT) and 0.5 μg/μl random primers (Promega), 10 mM dithiothreitol, 0.75 μl of dNTPs (10 mM dATP, 10 mM dCTP, 10 mM dGTP and 2 mM dUTP; Applied Biosystems), 30 U RNAsin (Promega) and 14 U of SuperScript II enzyme reverse transcriptase (Invitrogen) for 2 h at 42 °C. Lucidea™ Spike Mix Controls (Amersham-GE Healthcare Life Sciences) were added to the control and test samples prior to the labeling reaction. Subsequently, the mRNA was degraded with 0.08 N NaOH (Merck) and 40 mM EDTA (Sigma) for 30 min at 65 °C. The degradation was stopped with 0.17 M Tris-HCl, pH 7.5. Finally, the control and test samples were combined and purified using CyScrybeTM GFXTM columns (Amersham-GE Healthcare Life Sciences) following the manufacturer's protocol. Samples were added with 0.4 μg/μl of poly(dA) (Sigma) and 0.08 μg/μl of Cot-1 DNA (Invitrogen), lyophilized, resuspended in 20 μl of hybridization buffer (25% formamide, 2.98% SSC, 0.20% SDS, and Denhardt's 5X solution in water), and incubated at 55 °C for 3 min.

Microarray slides were blocked for 15 min at room temperature with 1% bovine serum albumin (BSA) (Sigma) preheated to 50 °C. Then, to denature the probes, the slides were incubated in boiling water for 5 min and fixed for 2 min with 95% ethanol at 4 °C. Labelled samples were hybridized on the microarrays using LifterSlip coverslips (Erie Scientific Company). Hybridization was carried out in a BioMixer II hybridizer (CapitalBio) at 55 °C for 16–20 h. After hybridization, the microarrays were washed with Microarray Wash Buffers A, B, and C (ArrayIt).

2.5.2. Microarray analysis

Microarrays were scanned with the ScanArray Express (PerkinElmer) using the ScanArray Express 3.0 software. Background-corrected data were normalized by the locally weighted scatterplot smoothing (LOWESS) method, based on the intensity of the Lucidea Universal ScoreCard control probes, and using an R script specifically developed for the Cholestchip. Normalised data were processed to eliminate the technical controls, data with intensity below the scanner's detection confidence limit, and inconsistent replicates. The arithmetic mean of the Cy5/Cy3 coefficients for each probe was Log_2-transformed (M value). $M \geq 0.85$ and $M \leq -0.85$ were considered significant changes. Next, data were subjected to cluster analysis (hierarchical cluster and k-means), using Euclidean distances, with the MultiExperiment Viewer software (Mev v3.1) [26]. Finally, we represented the changes in the cholesterol biosynthesis pathway using the PathwayExplorer software [27].

2.6. Real-time PCR assay

500 ng of total RNA, extracted with TRI Reagent (Sigma), were reverse transcribed with random hexamers using the PrimeScript RT reagent kit (Takara). Real-time PCR amplification was performed on a LightCycler 480 II using the SYBR Green I Master kit (Roche). The thermocycler protocol consisted of a preincubation step at 95 °C for 5 min, followed by 45 cycles at 95 °C for 10 s, 60 °C for 10 s, and 72 °C for 10 s. To prove that only one PCR product was amplified, melting curves were obtained with a temperature ramp of 65 °C to 97 °C with changes of 0.11 °C/s. Relative expression of each gene was determined by calculating the increase in CP (ΔCP), as previously described [28], corrected for the efficiency of the assay for each pair of primers, and normalized by the housekeeping control *RPLP0* (large ribosomal protein gene P0). The sequence of primers used in this study can be found in Supplementary Table 1.

2.7. LXRα luciferase reporter assay

Cells were transduced with a lentivirus (QIAGEN, Cignal Lenti Reporter Assay, CLS-7041L) to stably express the LXRα response elements upstream of a luciferase reporter gene, following the manufacturer's instructions. Transduced cells were selected performing a puromycin kill curve. As an internal control for normalization, cells were transduced with a constitutively expressing *Renilla* luciferase construct (QIAGEN, Cignal Lenti Reporter Assay, CLS-RHL). For the experiments, cells were treated as described above and lysed for dual luciferase reporter assay (Promega), following the manufacturer's instructions. Luminescence intensity was measured using a Sirius dual-injector luminometer (Berthold). LXRα activity was calculated as firefly/*Renilla* luciferase activity ratios after subtracting the background (non-transduced cells).

2.8. Western blot analysis

Cells were lysed in 20 mM Tris-HCl buffer, pH 8.0, containing 120 mM KCl, 1 mM dithiothreitol, 1 mM Na_2-EDTA, 2 mM EGTA, 0.1% Triton X-100, 0.5% Nonidet P-40, 1 mM benzamidine, 10 μg/ml antipain, 1 μg/ml leupeptin, 40 μg/ml aprotinin, 100 mM NaF, 20 mM sodium molybdate, 20 mM β-glycerophosphate, 2 mM sodium orthovanadate, 1 mM PMSF, and 1 μg/ml Caspase-3 inhibitor Ac-DMQD-CHO, for 20 min under stirring and then sonicated at 4 °C. Samples were passed 10 times through a 25-gauge needle and centrifuged at 10,000 x g for 10 min. Protein concentration was measured in the collected supernatant by the bicinchoninic acid method (BCA Protein Assay Kit, Pierce). Equal amounts of protein were subjected to SDS-PAGE, transferred to a nitrocellulose membrane and probed with antibodies directed against SREBP-2 (ab30682, Abcam). Bound antibodies were visualized using secondary antibodies conjugated to IRDye 800CW

(LI-COR). Fluorescence was detected using an Odyssey 9120 scanner (LI-COR Biosciences).

2.9. Microscopy

MOLT-4 cells were treated or not with the different SERMs. 2 h before finishing the treatments, 7×10^4 cells/cm^2 were seeded on glass coverslips previously treated with poly-D-lysine and the incubation continued at 37 °C to allow cell adhesion. For free cholesterol staining, cells were fixed with 4% paraformaldehyde for 5 min and exposed to filipin (35 μg/ml in PBS) for 1 h. Coverslips were mounted on slides using ProLong Gold antifade reagent. Cells were examined on an Olympus BX51 fluorescence microscope.

2.10. Statistical analysis

To evaluate the effects of SERMs and their interaction with other drugs, the two-way repeated measures ANOVA analysis was used. Post

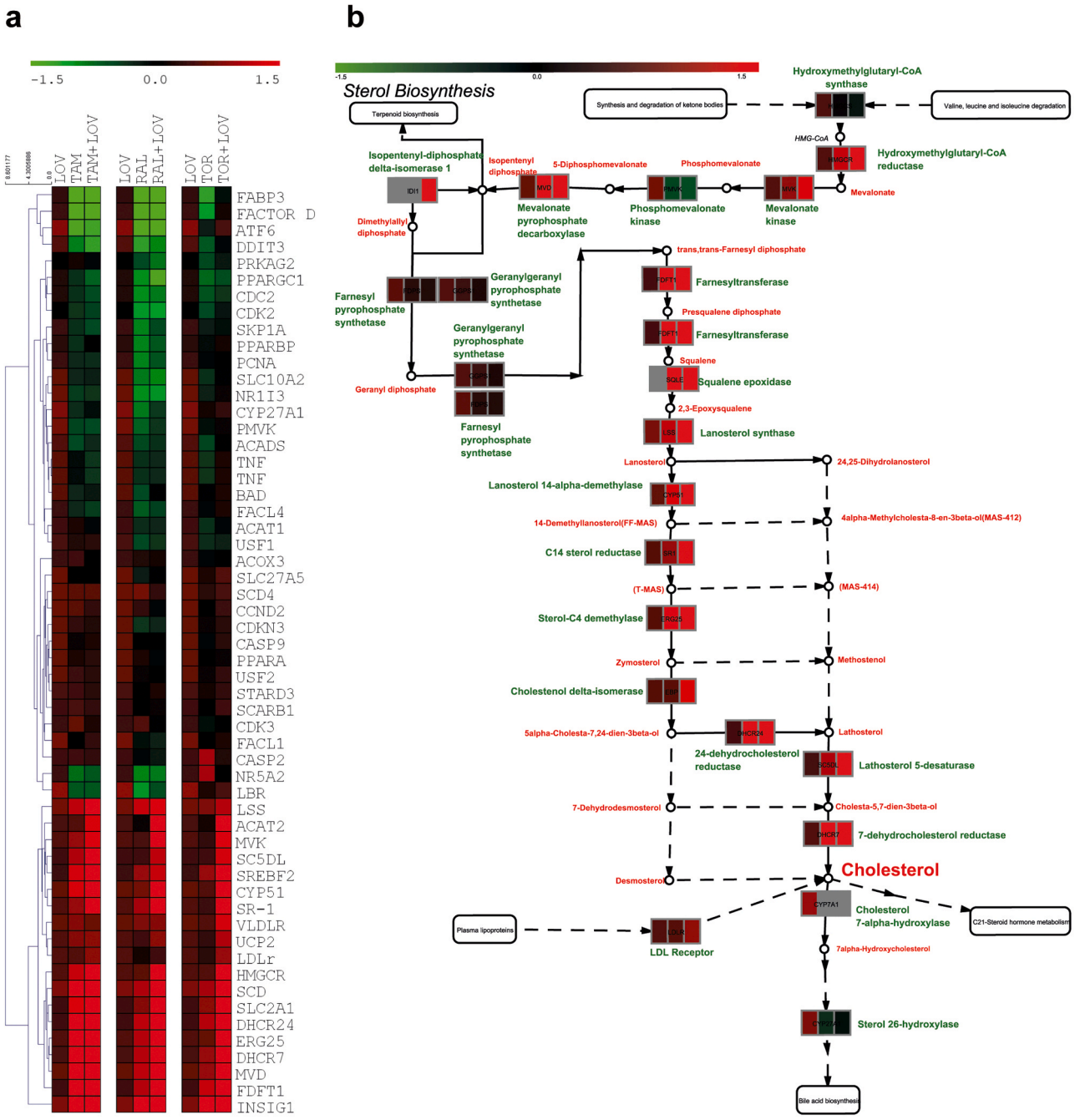

Fig. 1. Microarray analysis of gene expression patterns in MOLT-4 cells treated with SERMs or their combinations with LOV. Cells were treated with 10 μM SERMs, 1 μM LOV or their combinations in the presence of 120 μg/ml LDL-cholesterol for 16 h. (a) Hierarchical tree cluster with Euclidean Distances. Only genes whose expression changed in at least one condition in the two independent experiments were included in the analysis. The heatmap contains representative data, and each column belongs to a treatment and each row to a gene. Red indicates overexpression, green repression and black no change. (b) Effect of TAM and LOV and their combination on the expression of genes of the cholesterol biosynthesis pathway in MOLT-4 cells. Each box represents one of the enzymes of the pathway. Within each box, the result on the left corresponds to LOV, on the centre to TAM and on the right to the combination of both. One experiment of two independent experiments is represented. The increases in intensity of red and green correspond to increase and decrease of expression, respectively.

hoc multiple comparisons were performed by Student-Newman-Keuls test. The analyses were performed using the SigmaStat 2.0 software. $p \leq 0.05$ was defined as statistically significant.

3. Results

3.1. Tamoxifen, raloxifene, and toremifene increase the expression of SREBP target genes

To determine which genes involved in lipid metabolism changed their expression in response to treatment with SERMs, and to ascertain whether LOV could potentiate the effect of SERMs, we used the DNA focused microarray Cholestchip™. The lymphoblastic cell line MOLT-4 was incubated in medium supplemented with LPDS for 24 h and treated with TAM, RAL, or TOR combined or not with LOV and in the presence of LDL. After 16 h of treatment, gene expression was analysed. Of the 317 genes represented in the microarray, 119 genes (37.5%) were expressed under all the conditions. Of them, 56 (47%) changed significantly in at least one condition, with a cut-off M-value > 0.85 or < -0.85 (Fig. 1a). We performed a cluster analysis using the KMC method, with calculated K-means and Euclidean distances, and found four sets of genes with a similar response. The first cluster consisted of genes that increased their expression in response to SERMs and had a synergistic effect with LOV. The second cluster was similar to the previous one but with an unclear enhanced expression when combined with LOV. The data of these two groups are represented in Table 1. The third cluster consisted of genes repressed upon SERMs treatment (Table 2). Finally, the fourth cluster contained genes that showed erratic behaviour. It should be noted that we did not find differential effects between the SERMs amongst the genes analysed.

We found 19 genes overexpressed in all the conditions (Table 1), 16 of which were targets of the transcription factors SREBPs. Among them, the most represented were genes encoding enzymes of the cholesterol biosynthesis pathway, with 12 genes being upregulated. The effects of TAM on genes of the cholesterol biosynthesis pathway are represented in Fig. 1b. The effects of RAL and TOR on the cholesterol biosynthesis pathway are represented in Supplementary Figs. 1 and 2, respectively. These results suggest that SREBPs, particularly SREBP-2, which is the primary activator of cholesterol synthesis and LDL uptake genes, mediate many of the effects of SERMs on lipid metabolism genes. To confirm these results, we analysed, by real-time PCR, the expression of a set of representative SREBP-targeted genes: *LDLR*, *HMGCR*, *SCD*, *FDFT1* and *INSIG1*. Real-time PCR results correlated well with those of the microarray. SERMs increased the expression of these genes (Fig. 2a-e). The treatment with LOV alone did not modify gene expression, but when LOV was combined with any of the SERMs, the effect surpassed that of the SERM alone. Moreover, we analysed a set of non-SREBP target genes (*DGAT2*, encoding diacylglycerol O-acyltransferase 2; *ACADM*, encoding medium-chain specific acyl-coenzyme A dehydrogenase; and *HADHA*, encoding hydroxyacyl-coenzyme A dehydrogenase/3-ketoacyl-coenzyme A thiolase/enoyl-coenzyme A hydratase [trifunctional protein], alpha subunit) whose expression was detectable but did not change according to the microarray analysis. As shown in Fig. 2f-h, the quantitative expression of these genes was not significantly altered by the different treatments.

We wanted to know if the effects of the SERMs observed in MOLT-4

Table 1
Genes that increased their expression by microarray analysis following treatment with SERMs or their combinations with LOV in MOLT-4 cells. Data are M values, $M=\log_2$ (fluorescence treated sample / fluorescence control sample), from two independent experiments. SREBP target genes are in bold letters.

Gene	LOV	TAM	TAM+LOV	RAL	RAL+LOV	TOR	TOR+LOV	Name
LSS	1.68	2.27	3.32	2.33	2.68	1.69	2.19	Lanosterol synthase
	1.51	1.68	3.46	1.69	1.84	1.49	1.55	
ACAT2	1.43	1.45	2.53	1.10	2.87	1.13	3.94	Acetyl-coenzyme A acetyltransferase 2
	1.43	1.33	1.92	1.29	2.53	1.57	1.99	
MVK	1.45	1.88	2.68	1.40	3.51	1.27	2.68	Mevalonate kinase
	1.47	1.25	1.48	1.16	1.73	1.52	1.40	
SC5DL	1.37	2.08	3.46	1.36	2.22	1.37	2.43	Lanosterol 5-desaturase
	1.36	1.30	1.99	1.35	1.44	1.41	1.46	
SREBF2	1.33	2.46	3.53	1.92	2.33	1.92	3.07	Sterol regulatory element binding transcription factor 2
	1.16	1.57	1.73	1.35	1.53	1.60	1.47	
CYP51	1.54	2.57	4.17	1.66	3.14	1.48	3.58	lanosterol 14-alpha demethylase
	1.51	1.68	2.16	1.60	2.30	1.43	1.64	
SR-1	1.46	1.92	2.81	2.00	2.95	1.33	3.63	C14 sterol reductase
	1.62	1.57	2.03	1.41	2.19	1.32	1.36	
VLDLR	1.57	1.80	1.59	1.74	1.78	1.45	2.50	Very low density lipoprotein receptor
	1.55	1.62	1.72	1.29	1.48	1.58	1.28	
UCP2	1.46	1.78	1.85	1.37	1.83	1.29	2.11	Uncoupling protein 2
	1.37	1.17	1.45	1.09	1.39	1.39	1.32	
LDLR	1.44	1.47	1.85	1.11	1.38	1.22	1.88	Low density lipoprotein receptor
	1.38	1.41	1.89	1.23	1.29	1.23	1.12	
HMGCR	1.44	2.33	4.44	1.52	4.38	1.47	5.28	3-hydroxy-3-methylglutaryl-coenzyme A reductase
	1.39	1.77	2.73	1.55	3.05	1.42	1.72	
SCD	1.72	2.81	4.29	2.07	4.35	2.04	5.10	stearoyl-coenzyme A desaturase
	1.61	1.88	2.31	2.04	3.03	1.87	2.08	
SLC2A1	1.35	2.66	5.46	1.80	4.20	1.83	5.94	Solute carrier family 2
	1.56	1.77	2.71	1.73	3.12	1.97	2.30	
DHCR24	1.38	2.83	5.35	2.20	4.50	2.17	6.19	24-dehydrocholesterol reductase
	1.31	1.88	2.68	1.88	3.14	1.71	1.91	
ERG25	1.46	2.79	5.10	2.14	5.98	1.58	6.73	Sterol C4 demethylase
	1.69	1.68	2.77	1.87	3.39	1.59	2.03	
DHCR7	1.43	2.99	5.98	2.14	5.50	1.88	6.15	7-dehydrocholesterol reductase
	1.54	1.80	2.48	2.16	3.51	1.53	1.96	
MVD	1.56	2.85	5.24	2.22	6.96	1.91	5.74	Mevalonate (diphospho) decarboxylase
	1.52	1.74	2.57	2.01	3.51	1.40	1.59	
FDFT1	1.38	2.91	5.31	2.13	4.44	2.28	5.70	Farnesyl-diphosphate farnesyltransferase 1
	1.48	2.04	3.05	2.36	3.53	2.43	3.41	
INSIG1	1.71	5.58	12.64	3.97	10.70	4.35	12.38	Insulin induced gene 1
	1.78	2.87	4.23	3.58	5.28	2.41	3.46	

Table 2
Genes that decreased their expression by microarray analysis following treatment with SERMs or their combinations with LOV in MOLT-4 cells. Data are M values, M=$\log_2$ (fluorescence treated sample / fluorescence control sample), from two independent experiments.

Gene	LOV	TAM	TAM+LOV	RAL	RAL+LOV	TOR	TOR+LOV	Name
PRKAG2	1.01	1.11	0.97	0.70	0.56	0.67	0.88	Protein kinase, AMP-activated, gamma 2 non-catalytic subunit
	1.06	0.65	0.75	0.53	0.57	0.93	0.82	
PPARGC1	1.20	0.80	0.61	0.51	0.37	0.59	0.66	Peroxisome proliferative activated receptor, gamma, coactivator 1, alpha
	1.09	0.61	0.95	0.54	0.52	1.03	0.94	
CDC2	1.21	0.78	0.68	0.51	0.55	0.65	0.77	Cell division cycle 2, G1 to S and G2 to M
	1.04	0.67	0.73	0.61	0.64	0.89	0.78	
CDK2	0.03	-0.36	-0.46	-1.07	-1.1	-0.53	-0.16	Cyclin-dependent kinase 2
	0.18	-0.48	0.26	-0.68	-0.46	0.12	-0.18	

cells were exclusive of this lymphoid cell type, or if they could be reproduced in a cell type important in lipid metabolism, such as hepatocytes. For this, we used the HepG2 human hepatocyte cell line. The cells were treated following the same experimental design as with MOLT-4 cells. Results of microarrays assays in HepG2 cells paralleled those obtained in MOLT-4 cells. From the genes expressed in all the conditions (Supplementary Fig. 3a), there was an enrichment of SREBPs targets amongst the stimulated genes (Supplementary Table 2), the majority of them belonging to the cholesterol biosynthesis pathway. The effects of TAM, RAL, and TOR on this pathway are individually represented in Supplementary Figs. 3b, 4 and 5, respectively. Curiously, one of the most upregulated genes was *FABP1*, a fatty acid transporter that, although it has not been described as an SREBP target gene, had a similar behaviour to that of SREBP known targets. Repressed genes are summarised in Supplementary Table 3. To corroborate the results obtained by microarray analysis, we chose three representative upregulated genes, *FABP1*, *MVD,* and *ERG25*, and analysed their expression by real-time PCR (Supplementary Fig. 6). As with MOLT-4, real-time PCR results correlated with those of the microarray. Thus, it was confirmed that *FABP1* responded similarly to known targets of SREBP (Supplementary Fig. 6a).

3.2. SERMs modulation of SREBP and LXR target gene expression depends on LDL-cholesterol uptake

Since SERMs interfere with the lysosomal trafficking of cholesterol [13,14,20], we wanted to explore whether SERMs could also affect the expression of lipid metabolism genes when cholesterol is administered to cells in a non-lipoprotein form. For this, we used a cholesterol and methyl-cyclodextrin complex (CholMCD), widely used to efficiently transfer cholesterol to cells independently of the endocytic pathway [23, 29–31]. We incubated the MOLT-4 cells with the different SERMs combined or not with LOV in the presence of LDL or CholMCD. We analysed the expression of *LDLR, HMGCR, and INSIG1*, as representatives of the SREBP-target genes, and *ABCA1*, as the main representative of the LXR-target genes. The amount of cholesterol added with CholMCD was adjusted so that the magnitude of the effect on the SREBP targets was comparable to that produced by LDL. As references, we included two conditions without cholesterol in the medium, containing only LPDS or LPDS plus LOV.

As expected, the addition of LDL to the medium decreased the expression of the SREBP target genes *LDLR*, *HMGCR,* and *INSIG1*, compared to the condition with LPDS alone (Fig. 3a-c). The three SERMs prevented the repression caused by the addition of LDL. LOV treatment did not change the expression of these genes versus LDL alone, suggesting the predominance of the repressive effect of LDL. However, when LOV was combined with any of the SERMs, the expression of the three genes increased synergistically. When cells were supplied with CholMCD, we observed a decrease in the expression of the SREBP target genes relative to LPDS, similar to that in cells exposed to LDL. But, contrary to what happened with LDL, the SERMs, in combination or not with LOV, did not prevent the repression caused by the addition of CholMCD (Fig. 3a-c). These results indicate that SERMs only interfere with the SREBP-mediated feed-back effect of cholesterol when cells are supplied with LDL-cholesterol, but not when supplied with non-lipoprotein cholesterol.

The expression of the LXR target gene *ABCA1* did not change upon LDL addition in comparison to LPDS alone (Fig. 3d). The addition of the different SERMs caused intense repression of *ABCA1* expression relative to its control condition without SERM, independently of the combination with LOV. Contrary to the lack of response of the SREBP target genes, in the presence of CholMCD, SERMs repressed the expression of *ABCA1*, although to a lesser extent than with LDL (Fig. 3d).

To determine if these effects were exclusive to MOLT-4 cells we reproduced the previous experiment in HepG2 cells. In these cells, we studied the *SCD* gene, also a target for SREBP, instead of *INSIG1*, which was expressed at very low levels. The effects of SERMs on SREBP targets (Supplementary Fig. 7a–c) were similar to those obtained in MOLT-4 cells. Curiously, in HepG2 cells, SERMs or their combinations with LOV had no effect on the expression of *ABCA1* in the presence of LDL nor in that of CholMCD (Supplementary Fig. 7d).

To further study the role of cholesterol from different sources, we analysed a third cell line, human THP-1 monocytes differentiated to macrophages (Supplementary Fig. 8). The effect of SERMs on SREBP target genes was analogous to those in MOLT-4 and HepG2 cells. Similar to HepG2, the expression of *ABCA1* did not vary in response to SERMs (Supplementary Fig. 8d).

3.3. LDL-cholesterol but not MCD-cholesterol accumulates within the cells

In light of our results showing different effects of SERMs on gene expression depending on the cholesterol source, we assessed the effects of SERMs and LOV on free cholesterol distribution when LDL or CholMCD were supplied to MOLT-4 cells as before. Filipin staining revealed that, in the presence of LDL, cells treated with SERMs and their combinations with LOV accumulated cytoplasmic vesicles containing free cholesterol in a distribution compatible with late endosomes/lysosomes localisation, while LOV alone produced no effect (Fig. 4). When the cells were incubated with CholMCD alone the staining was similar to that of cells treated with LDL alone (Fig. 4). None of the SERMs or their combinations with LOV induced accumulation of free cholesterol in the presence of CholMCD. These results correlate with those of gene expression and confirm that SERMs only significantly affect cholesterol processed through the endosomal route.

3.4. SERMs induce SREBP-2 processing and prevent LXR activation

Gene expression studies suggest that the main effects of SERMs on the regulatory response to LDL may involve the SREBP-2 and LXR pathways, both requiring the lysosomal export of LDL-derived cholesterol for them to be inhibited and activated, respectively. Thus, we directly assessed the processing of SREBP-2 and the activation of LXR. We selected HepG2 cells for this part of the study because of the relevance of hepatocytes to cholesterol metabolism.

First, we evaluated the processing of SREBP-2 in cells treated with TAM in the presence of LDL for different incubation times. To perform

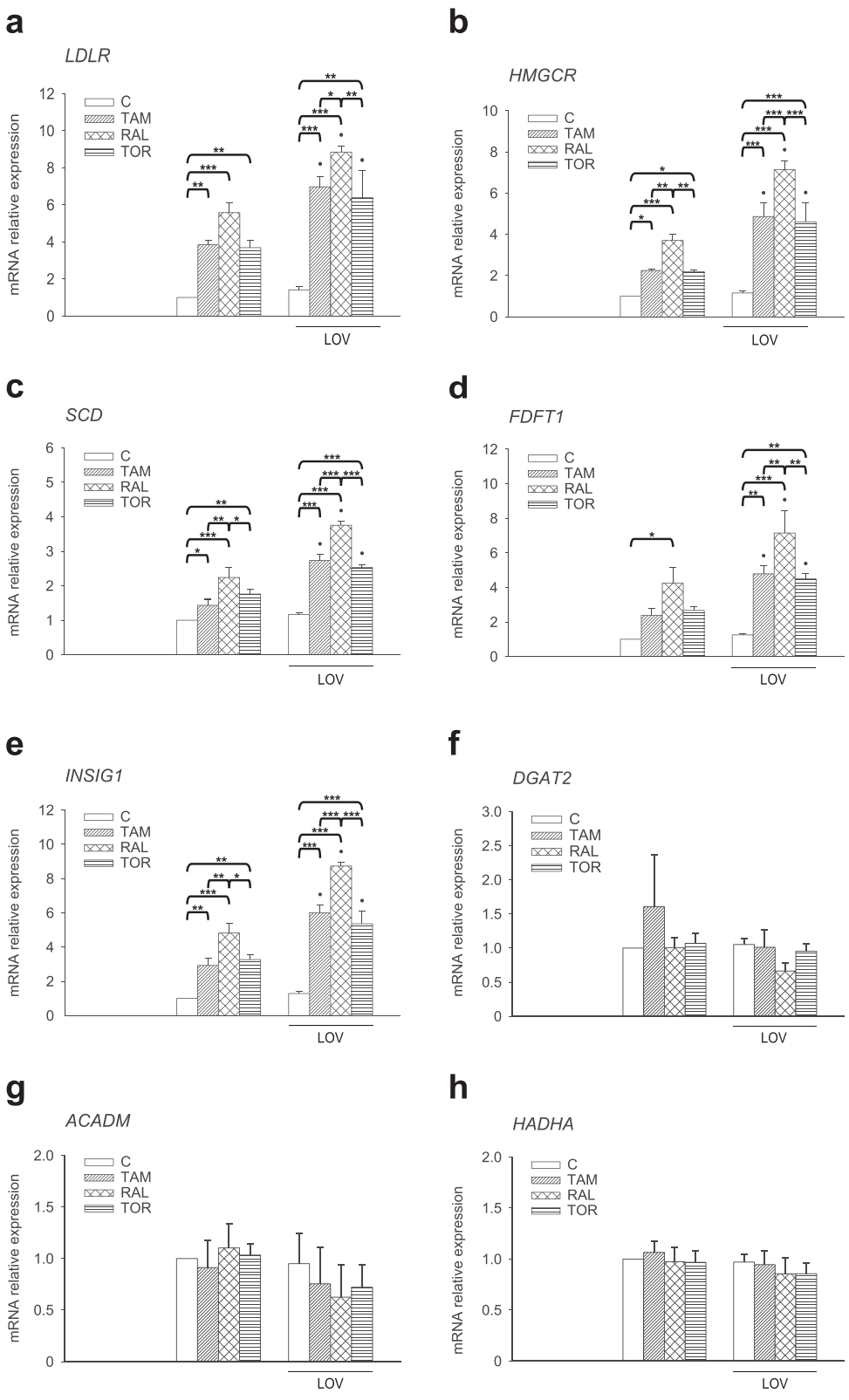

Fig. 2. Validation of microarray expression results of selected genes by real-time PCR in MOLT-4 cells. Cells were treated with 10 μM SERMs, 1 μM LOV or their combinations in the presence of 120 μg/ml LDL-cholesterol for 16 h. Data represent the mean ± SEM of three independent experiments, expressed as the relative amount of mRNA compared to the control (C) condition in the absence of LOV (set to 1). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$; •$p < 0.05$ compared to the same condition without LOV.

these experiments, we used a combination of 0.1% hydroxypropyl-cyclodextrin (HPCD) and 1 μM LOV for 2 h, a condition that causes rapid cholesterol depletion and, consequently, stimulates SREBP-2 processing and the appearance of the nuclear form of SREBP-2 (n-SREBP-2). TAM produced an increase in the processing of SREBP-2, although this effect was transitory (Fig. 5a). At 13 h a moderate increase in the proportion of n-SREBP-2 was observed. At 16 h, the precursor form of SREBP-2 (p-SREBP-2) was nearly absent and the amount of n-SREBP-2 reached a maximum. Finally, at 19 h the effect ceased. Based on these data, we focused on the 16 h treatment to extend the study to RAL and TOR and their combination with LOV. As expected, given the presence of LDL in the medium, in the control condition almost

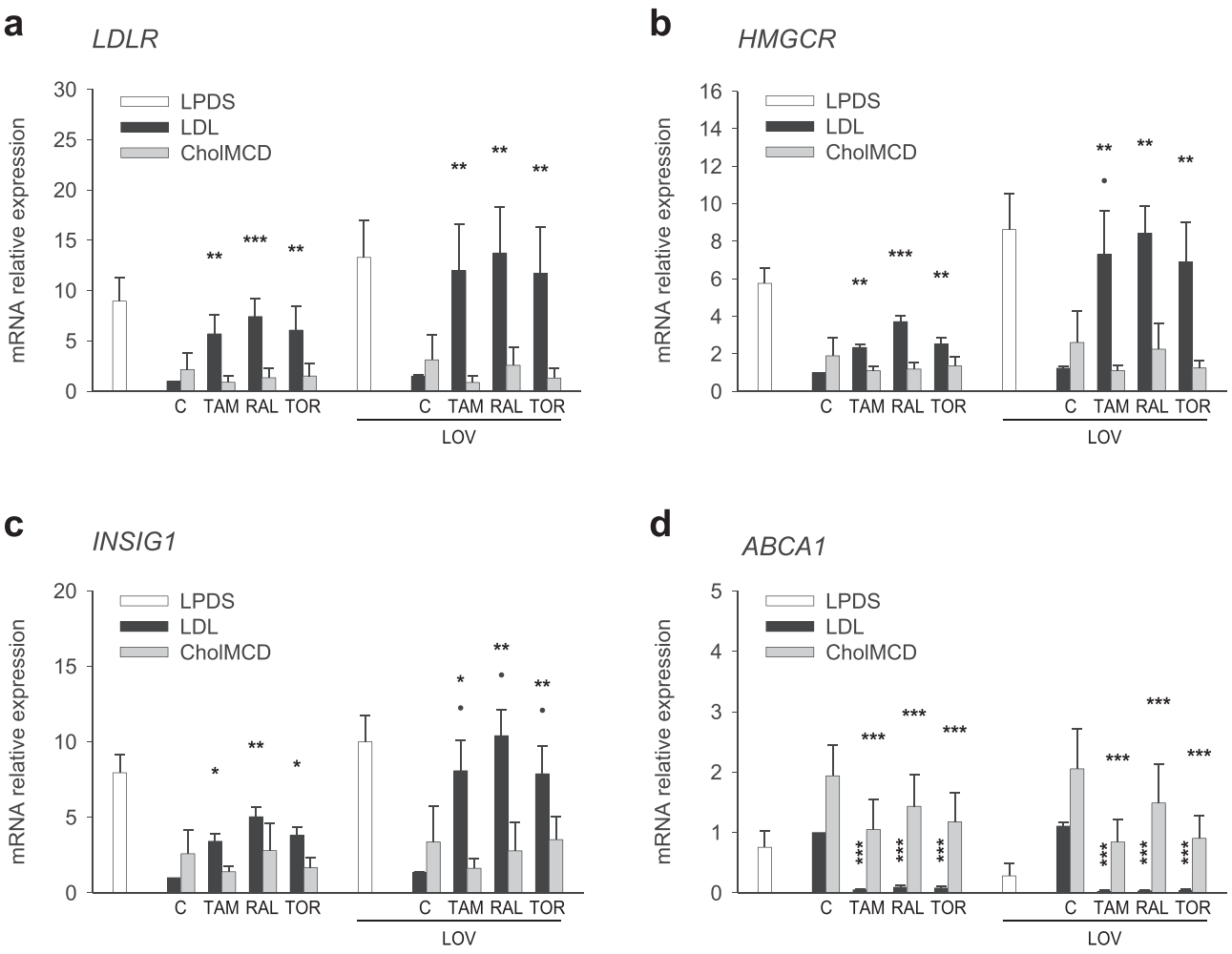

Fig. 3. Effect of SERMs and their combinations with LOV on the expression of selected genes in MOLT-4 cells. Cells were treated with 10 μM SERMs, 1 μM LOV or their combinations in the presence of 120 μg/ml LDL-cholesterol or 1.2 μg/ml cholesterol complexed with methyl-cyclodextrin (CholMCD). Alternatively, cells were incubated in the absence of cholesterol (LPDS) and SERMs as a reference. Data represents the mean ± SEM of three independent experiments, expressed as the relative amount of mRNA compared to the control (C) condition in the absence of LOV (set to 1). Results were analysed using the Two-Way Repeated Measures Anova test, and the Student-Newman-Keuls test post-hoc. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared to the same condition without any SERM (C); • $p < 0.05$ compared to the same condition without LOV.

all SREBP-2 was unprocessed (Fig. 5b). The addition of LOV alone did not increase the processing of SREBP-2 compared to the control condition. The addition of the SERMs increased the processing of SREBP-2, and the combination with the LOV produced a further increase.

LXR activation was studied using a luciferase reporter gene construction containing LXRE in its promoter. HepG2 cells were treated with TAM or remained untreated, in the presence of LDL, and LXR activity was measured at different time points. From 8 h of treatment, control cells, with only LDL, showed increased LXR activity that peaked at 16 h (Fig. 5c). TAM prevented the activation of LXR by LDL. Next, we assessed the effects of TAM, RAL, and TOR and their combinations with LOV on LXR activation at 16 h. The three SERMs reduced LXR activation versus the control condition, with LDL alone (Fig. 5d). LOV slightly reduced LXR activity. The addition of LOV in combination with each SERM did not produce any additional effect. Moreover, we evaluated the effect of SERMs in the presence of LDL and the LXR activator T0901317. As expected, T0901317 increased LXR activity relative to the control, and only RAL could slightly oppose to this effect. Finally, when cells were incubated with CholMCD, LXR activity increased moderately. The SERMs prevented this activation, but to a much lower extent than in the presence of LDL (Fig. 5d).

Additionally, we assessed the effect of SERMs on LXR activation in cholesterol-loaded macrophages. For this, we treated THP-1 macrophages with the SERMs in the presence of acetylated LDL (AcLDL), which is actively taken up by macrophages. The capacity of macrophages to activate LXR and increase cholesterol efflux is paramount to prevent cholesteryl ester deposition and the formation of foamy macrophages like those that accumulate in atheroma plaques [32]. Virtually identical responses to the different experimental conditions were obtained in THP-1 macrophages compared with those in HepG2 cells (Supplementary Fig. 9a and b). Overall, SERMs efficiently suppress the LDL-induced transcriptional activity of LXR.

3.5. Estrogen receptor-independent effects of SERMs

Given that SERMs are ligands of ERs, we evaluated the role of these receptors in the effects of SERMs. First, we measured the expression of the genes *ESR1* and *ESR2,* coding for ERα and ERβ, respectively. Expression of both *ESR1* and *ESR2* in HepG2 and THP-1 cells was undetectable (Ct > 40). In MOLT-4 cells, *ESR1* mRNA was also undetectable and *ESR2* mRNA was found in very small amounts (Ct = 30). These findings suggest that ERs do not mediate the observed effects of SERMs. To confirm this, we compared the responses to SERMs and 17β-estradiol (E2) in MOLT-4 cells to those in MCF-7 cells, a mammary gland ERα-positive cell line. The genes selected were *LDLR*, as a representative SREBP-2 target, and *BCL2*, *ESR1,* and *ESR2*, as ERs targets. *LDLR*

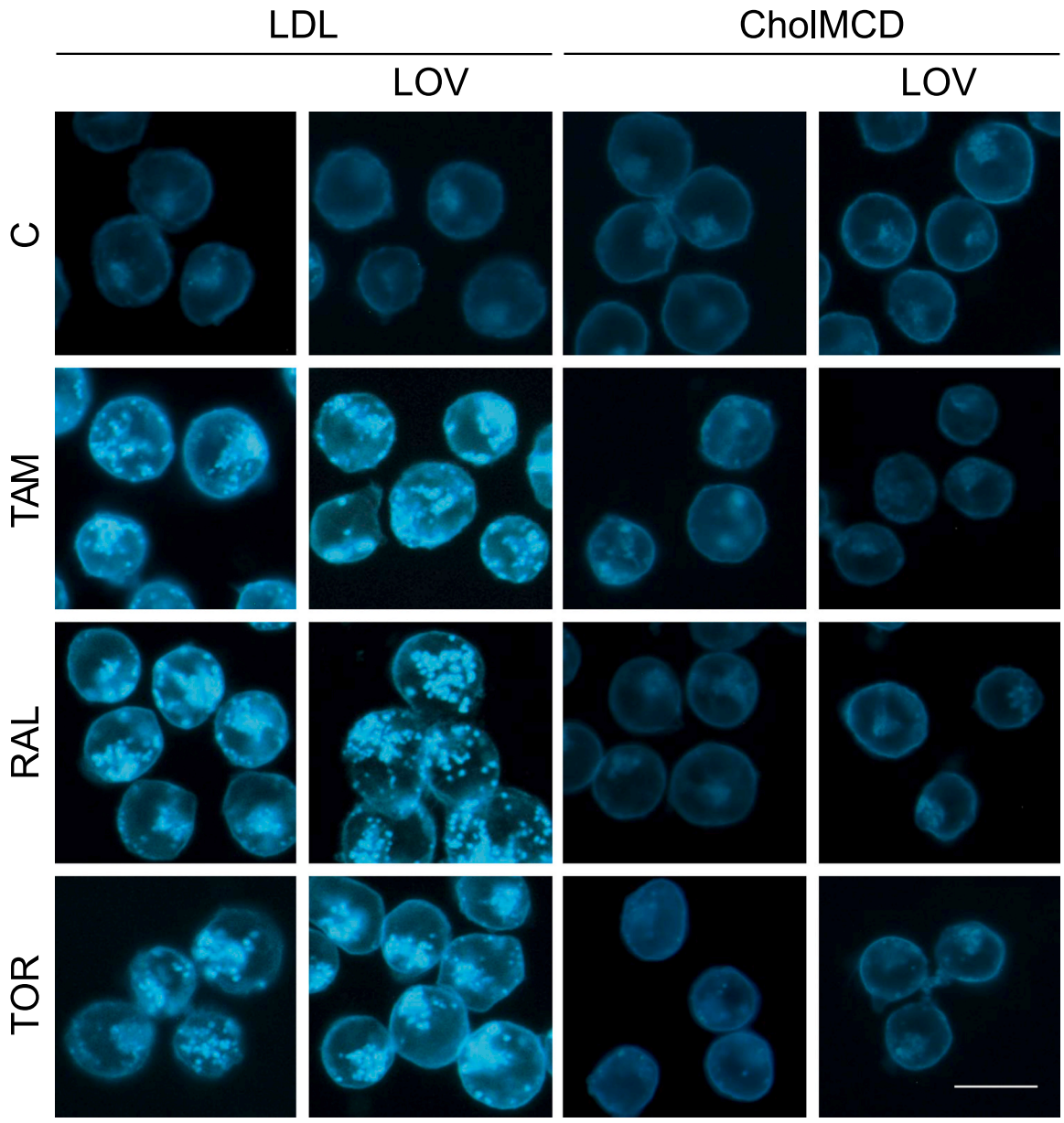

Fig. 4. Effect of SERMs and their combinations with LOV on intracellular distribution of free cholesterol in MOLT-4 cells. Cells were treated with 10 μM SERMs, 1 μM LOV or their combinations in the presence of 120 μg/ml LDL-cholesterol or 1.2 μg/ml cholesterol complexed with methyl-cyclodextrin (CholMCD) for 16 h. Results correspond to a representative experiment of two independent experiments. Scale bar: 20 μm.

expression did not change upon the addition of E2, but it did in response to SERMs in both cell lines (Fig. 6a). Consistently with the expression of ERα, *BCL2* expression increased in MCF-7, but not in MOLT-4 cells in response to E2 (Fig. 6b). The SERMs did not modify the expression of *BCL2* in MOLT-4 nor in MCF-7 cells. In MCF-7 cells, *ESR1* expression was inhibited only by E2 (Fig. 6c). The amount of *ESR2* mRNA did not significantly change when MOLT-4 cells were treated with E2 or SERMs compared to untreated cells (Fig. 6d). These results indicate that the effects of SERMs on the SREBP and LXR pathways are independent of the ERs.

4. Discussion

In this study, we demonstrate that the SERMs TAM, RAL and TOR influence the activity of the lipid metabolism master regulators SREBP and LXR by an ER-independent pathway and in association with the interference with the trafficking of LDL-derived cholesterol via the endosomal pathway.

Microarray assays indicated that SREBP-2 targeted gene expression is increased by SERMs. In line with this, SREBP-2 processing is stimulated by SERMs, an effect that likely results from the blockade of the exit of LDL-derived cholesterol from lysosomes, thereby preventing the cholesterol-mediated inhibition of SREBP-2 processing. Moreover, the effect of SERMs on SERBP-2 processing and expression of its target genes is synergistic with that of LOV. This synergy can be the consequence of the disruption of LDL-cholesterol trafficking by SERMs combined with the inhibition of cholesterol biosynthesis by LOV. Although SERMs reduce cholesterol biosynthesis, they are less potent than LOV [13] and, hence, *de novo* synthesized cholesterol can still reach the endoplasmic reticulum and retain some SREBP-2. When only LOV is administered, it does not produce any effect, since the repressive effect of LDL-cholesterol prevails over the effect of cholesterol biosynthesis inhibition on SERBP-2 processing. Yet, when LOV is combined with any of the SERMs, the arrival of LDL cholesterol to the endoplasmic reticulum

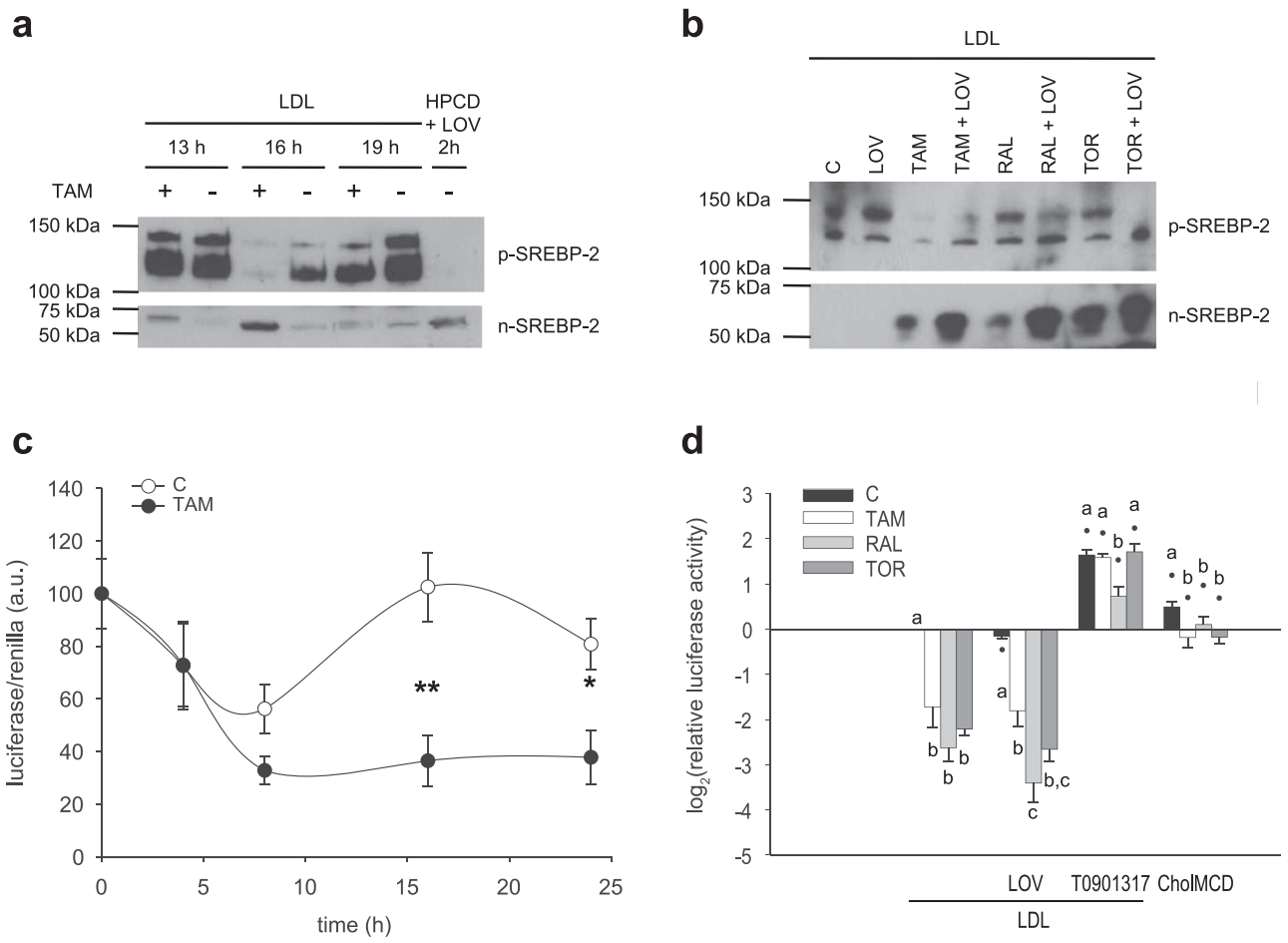

Fig. 5. Effect of SERMs and their combinations with LOV on SERBP-2 and LXR activation in HepG2 cells. (a) Time-dependent effect of 10 μM TAM on SREBP-2 processing in the presence of 120 μg/ml LDL-cholesterol. As a positive control for SREBP-2 processing, cells were treated with 0.1% hydroxypropyl-cyclodextrin (HPCD) plus 1 μM lovastatin (LOV) for 2 h. (b) Effect of 10 μM SERMs, 1 μM LOV or their combinations on SREBP-2 processing at 16 h of treatment in the presence of 120 μg/ml LDL-cholesterol. (c) Time-dependent effect of 10 μM TAM on LXR transcriptional activity in the presence of 120 μg/ml LDL-cholesterol. Data represent the mean ± SEM of five independent experiments; au, arbitrary units. (d) Effect of 10 μM SERMs combined or not with 1 μM LOV or 1 μM T0901317 on LXR transcriptional activity at 16 h of treatment in the presence of 120 μg/ml LDL-cholesterol. The effect of SERMs in the presence of 30 μg/ml of cholesterol complexed with methyl-cyclodextrin (CholMCD) was also assessed. Results are expressed as $\log_2$ and relative to the activity in the condition with LDL alone, and represent the mean ± SEM of four independent experiments. Results were analysed using the Two-Way Repeated Measures Anova test, and the Student-Newman-Keuls test post-hoc. * $p < 0.05$, ** $p < 0.01$. Bars not sharing any letter are statistically different to the conditions only differing in the SERM treatment ($p < 0.05$); •$p < 0.05$ compared to the same SERM condition with only LDL (d). C, control (no SERM).

as well as cholesterol biosynthesis are blocked, resulting in a synergistic effect on SREBP-2 processing and expression of target genes.

MCD complexes with the fluorescent cholesterol analog dehydroergosterol (DHE) have previously been used for intracellular cholesterol trafficking studies, and have shown that they deliver DHE directly to the plasma membrane and, from there, DHE is transported to recycling endosomes or lipid droplets via non-vesicular transport, with no involvement of late endosomes or lysosomes [30,31]. SERMs did not prevent CholMCD-induced inhibition of SREBP-2 target gene expression. This correlates with the absence of free cholesterol deposits in cells treated in the same conditions. This result contrasts with the blockade of cholesterol trafficking and the consequent accumulation of free cholesterol in late endosomes/lysosomes produced by SERMs in the presence of LDL, previously described by our group [13,14,20] and confirmed in this study.

The interference with intracellular cholesterol trafficking produced by SERMs also reduced LXR activity. This may be a consequence of the inability for LDL-derived cholesterol to leave late endosomes/lysosomes and, thus, form oxysterols, which bind to LXR and activate the transcription of genes involved in the efflux of cholesterol, such as *ABCA1* and *ABCG1* [33]. Nonetheless, in the present study, the reduction in *ABCA1* expression was observed in MOLT-4 cells, but not in HepG2 cells or THP-1 macrophages, reflecting a cell-type-dependent regulation. The reduced LXR activity observed in THP-1 macrophages treated with SERMs in the presence of AcLDL agrees with our previous report in which ABCA1 and ABCG1 protein expression were repressed in these conditions [20]. Here, in order to compare different cell types, we assessed *ABCA1* expression in the presence of native LDL. The differential effect on *ABCA1* expression in THP-1 macrophages may lie in the efficiency of uptake of each LDL type, much lower for native than for chemically modified LDL [34]. In HepG2 cells, the lack of effect of SERMs on *ABCA1* expression may lie in the fact that *ABCA1* in hepatocytes is positively regulated by both SREBP-2 and LXR [35]. In this case, the inactivation of LXR might be compensated by the activation of SREBP-2. Unexpectedly, in MOLT-4 cells, SERMs reduced the expression of *ABCA1* also when cholesterol was supplied complexed with MCD, although to a much lesser extent than with LDL. A possible explanation may be the SREBP-induced expression of miR-33, encoded by an intron of *SREBF2* and which targets *ABCA1* mRNA [36]. As observed herein, the SERMs also impaired LXR transcriptional activity when CholMCD was added to cells, suggesting that these drugs are able to prevent LXR activation also through mechanisms independent of endolysosomal

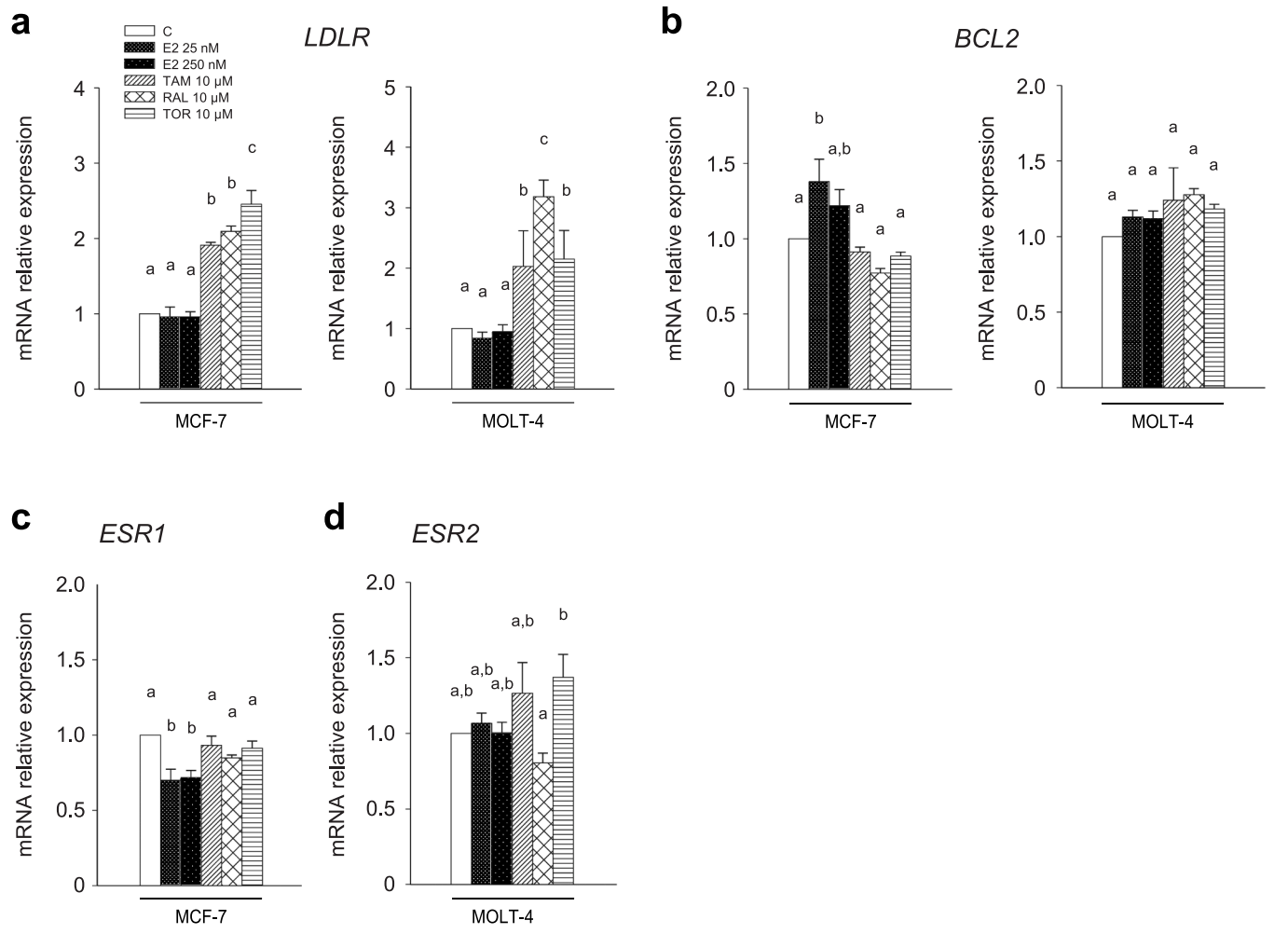

Fig. 6. Effect of E2 and SERMs on the expression of *LDLR, BCL2, ESR1* and *ESR2* genes in MCF-7 and MOLT-4 cells. Cells were treated or not (C) with the indicated concentrations of SERMs or E2 for 16 h in the presence of 120 μg/ml LDL-cholesterol. Data represent the mean ± SEM of three and four independent experiments for MOLT-4 and MCF-7 cells, respectively, expressed as the relative amount of mRNA compared to the control (C) condition (set to 1). Results were analysed using the Repeated Measures Anova test, with Student-Newman-Keuls post-hoc. Bars not sharing any letter are statistically different ($p < 0.05$).

cholesterol trafficking. This might relate to the inhibition of cholesterol biosynthesis [13,15,16] or the accumulation of 5,6-epoxycholesterols, of which the 5,6α diastereoisomer can modulate LXR activity and display agonist or antagonist effects in a cell and gene context-dependent manner [37,38].

As stated above, the most numerous set of genes with increased expression in response to SERMs was related to cholesterol biosynthesis and uptake, mainly targets of SREBP-2. However, these drugs also increased the expression of some SREBP-1c targets, such as *SCD* and *INSIG1*. *SCD* encodes stearoyl-coenzyme A desaturase, involved in lipogenesis [1]. This suggests that SERMs could stimulate lipogenesis in vivo and thus contribute to the increased incidence of hepatic steatosis described for TAM and, to a lesser extent, for RAL and TOR [39,40]. This mechanism could also be involved in the hypertriglyceridemia described in some patients treated with TAM [41]. Nonetheless, the poor representation of SREBP-1c target genes among the SERM-stimulated genes suggests that this transcription factor is not so markedly affected by the lysosomal cholesterol trafficking blockade. This effect can be explained by the dual gene expression regulation of SREBP-1c by LXR and SREBP-1 itself [41]. In this sense, an increase in SREBP-1c expression, due to reduced levels of cholesterol in the endoplasmic reticulum, could be compensated by a direct downregulation of LXR activity [42,43]. These results are in line with the interdependence and crosstalk between SREBP-1, SREBP-2, and LXR in order to maintain lipid homeostasis [44].

In this work, we showed that ERs are not involved in the effect of SERMs on SERBP-2 and LXR-mediated gene expression. A line of evidence is the lack of expression of ER genes in HepG2 and THP-1 cells and the marginal expression of only *ESR2* in MOLT-4 cells. Furthermore, in MOLT-4 cells, E2 had no effect on the expression of SREPB-2 or ERs target genes. These results contrast with those in MCF-7 cells in which the expression of *BCL2*, an ERα target gene, increased and that of *ESR1* decreased, due to ERα-mediated negative feedback, by treatment with E2, in agreement with other reports [45,46]. These results agree with our previous studies demonstrating that ERs do not mediate the SERMs-induced inhibition of LDL-cholesterol trafficking and efflux in macrophages [20] nor the increase in LDL uptake [14]. Although it has been described that TAM can stimulate *LDLR* expression by HepG2 cells through ERα, this was observed in ERα-transfected cells, whereas in non-transfected cells both TAM and E2 failed to activate transcription [47].

The effects of SERMs on intracellular cholesterol trafficking are common to a set of molecules that, like TAM, RAL, and TOR, are cationic amphiphilic drugs with lysosomotropic properties, that is, they accumulate in lysosomes [48]. The lipophilic characteristics in their structures allow them to passively diffuse through the lysosomal membrane when the molecule is in its basic non-ionized form, which is favoured by a concentration gradient [49]. At lysosomal acidic pH, the base is protonated and cannot leave this compartment, a process called pH

partitioning or ion trapping [48]. The best known of this class of compounds is U18666A [50], which, like SERMs, interferes with the endosomal trafficking of cholesterol, producing a cellular phenotype that resembles that caused by Niemann-Pick disease type C. There are other compounds with similar chemical characteristics, such as certain antipsychotics, antidepressants and antifungals, that also cause cholesterol accumulation in late endosome/lysosomes [51–54]. Some of these antipsychotics and antidepressants have been shown to stimulate SREBP activation and target gene expression [53,55–57].

The ability of TAM, RAL, and TOR to interfere with endosomal trafficking has been effectively used, in vitro, to inhibit Ebola virus entry and infection [51,58–60]. More studies are needed to explore the possibility of using these SERMs in the treatment of different viral infections. The endosome-related effects of SERMs can give to these widely used drugs a new clinical purpose beyond the ER pathway.

5. Conclusions

TAM, RAL, and TOR inhibit intracellular trafficking of LDL-derived cholesterol. Consequently, they prevent the regulatory effect of LDL over SREBP-2, thus increasing the processing of SREBP-2 and the expression of its target genes. This effect is synergistic when SERMs are combined with LOV. Furthermore, SERMs oppose to the activation of LXR in response to cholesterol overload. The molecular mechanism involved in these actions is independent of ERs. Our findings highlight the impact of SERMs on lipid homeostasis regulation beyond their actions as ER modulators, and are consistent with the changes in the lipid profile of patients taking this medication.

Funding sources

This work was supported by Ministerio de Ciencia, Innovación y Universidades (grant RTI2018-098113-B-I00), Plan Estatal de Investigación Científica y Técnica y de Innovación 2017–2020, Spain; European Regional Development Fund (ERDF/FEDER); European Cooperation in Science and Technology (COST) action BM0904, and Program ALIBIRD (grant S2013/ABI-2728), Comunidad de Madrid, Spain. CIBEROBN and CIBERDEM are initiatives of the Instituto de Salud Carlos III, Spain.

Conflict of interest statement

None.

Acknowledgements

We thank Lorena Crespo, Alejandra Quintero, Gema de la Peña and Nuria Crcoles for excellent technical assistance. M.A.L. is Emeritus of the Servicio Madrileño de Salud (Comunidad Autónoma de Madrid).

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.biopha.2021.111871.

References

[1] J.D. Horton, J.L. Goldstein, M.S. Brown, SREBPs: activators of the complete program of cholesterol and fatty acid synthesis in the liver, J. Clin. Invest. 109 (9) (2002) 1125–1131.
[2] M.S. Brown, J.L. Goldstein, Cholesterol feedback: from Schoenheimer's bottle to Scap's MELADL, J. Lipid Res. 50 (2009) S15–27.
[3] A. Chawla, J.J. Repa, R.M. Evans, D.J. Mangelsdorf, Nuclear receptors and lipid physiology: opening the X-files, Science 294 (5548) (2001) 1866–1870.
[4] M.B. Patel, N.A. Oza, I.S. Anand, S.S. Deshpande, C.N. Patel, Liver X receptor: a novel therapeutic target, Indian J. Pharm. Sci. 70 (2) (2008) 135–144.
[5] L.J. Millatt, V. Bocher, J.C. Fruchart, B. Staels, Liver X receptors and the control of cholesterol homeostasis: potential therapeutic targets for the treatment of atherosclerosis, Biochim. Biophys. Acta 1631 (2) (2003) 107–118.
[6] B. Wang, P. Tontonoz, Liver X receptors in lipid signalling and membrane homeostasis, Nat. Rev. Endocrinol. 14 (8) (2018) 452–463.
[7] C.K. Osborne, H. Zhao, S.A. Fuqua, Selective estrogen receptor modulators: structure, function, and clinical use, J. Clin. Oncol. 18 (17) (2000) 3172–3186.
[8] U. Gundimeda, Z.H. Chen, R. Gopalakrishna, Tamoxifen modulates protein kinase C via oxidative stress in estrogen receptor-negative breast cancer cells, J. Biol. Chem. 271 (23) (1996) 13504–13514.
[9] M. Poirot, S. Silvente-Poirot, R.R. Weichselbaum, Cholesterol metabolism and resistance to tamoxifen, Curr. Opin. Pharmacol. 12 (6) (2012) 683–689.
[10] S. Silvente-Poirot, M. Poirot, Cholesterol and cancer, in the balance, Science 343 (6178) (2014) 1445–1446.
[11] V.C. Jordan, SERMs: meeting the promise of multifunctional medicines, J. Natl. Cancer Inst. 99 (5) (2007) 350–356.
[12] J.H. Pickar, T. MacNeil, K. Ohleth, SERMs: progress and future perspectives, Maturitas 67 (2) (2010) 129–138.
[13] Y. Suarez, C. Fernandez, D. Gomez-Coronado, A.J. Ferruelo, A. Davalos, J. Martinez-Botas, M.A. Lasuncion, Synergistic upregulation of low-density lipoprotein receptor activity by tamoxifen and lovastatin, Cardiovasc. Res. 64 (2) (2004) 346–355.
[14] F. Cerrato, M.E. Fernandez-Suarez, R. Alonso, M. Alonso, C. Vazquez, O. Pastor, P. Mata, M.A. Lasuncion, D. Gomez-Coronado, Clinically used selective oestrogen receptor modulators increase LDL receptor activity in primary human lymphocytes, Br. J. Pharmacol. 172 (5) (2015) 1379–1394.
[15] H. Gylling, S. Pyrhonen, E. Mantyla, H. Maenpaa, L. Kangas, T.A. Miettinen, Tamoxifen and toremifene lower serum cholesterol by inhibition of delta 8-cholesterol conversion to lathosterol in women with breast cancer, J. Clin. Oncol. 13 (12) (1995) 2900–2905.
[16] B. Kedjouar, P. de Medina, M. Oulad-Abdelghani, B. Payre, S. Silvente-Poirot, G. Favre, J.C. Faye, M. Poirot, Molecular characterization of the microsomal tamoxifen binding site, J. Biol. Chem. 279 (32) (2004) 34048–34061.
[17] A.M. Dnistrian, M.K. Schwartz, E.J. Greenberg, C.A. Smith, D.C. Schwartz, Effect of tamoxifen on serum cholesterol and lipoproteins during chemohormonal therapy, Clin. Chim. Acta 223 (1–2) (1993) 43–52.
[18] P.D. Delmas, N.H. Bjarnason, B.H. Mitlak, A.C. Ravoux, A.S. Shah, W.J. Huster, M. Draper, C. Christiansen, Effects of raloxifene on bone mineral density, serum cholesterol concentrations, and uterine endometrium in postmenopausal women, New Engl. J. Med. 337 (23) (1997) 1641–1647.
[19] D. Gomez-Coronado, M.A. Lasuncion, J. Martinez-Botas, M.E. Fernandez-Suarez, Role of cholesterol metabolism in the anticancer pharmacology of selective estrogen receptor modulators, Semin. Cancer Biol. 73 (2021) 101–115.
[20] M.E. Fernandez-Suarez, J.C. Escola-Gil, O. Pastor, A. Davalos, F. Blanco-Vaca, M. A. Lasuncion, J. Martinez-Botas, D. Gomez-Coronado, Clinically used selective estrogen receptor modulators affect different steps of macrophage-specific reverse cholesterol transport, Sci. Rep. 6 (2016) 32105.
[21] J. Leignadier, F. Dalenc, M. Poirot, S. Silvente-Poirot, Improving the efficacy of hormone therapy in breast cancer: the role of cholesterol metabolism in SERM-mediated autophagy, cell differentiation and death, Biochem. Pharmacol. 144 (2017) 18–28.
[22] D. Calvo, D. Gomez-Coronado, Y. Suarez, M.A. Lasuncion, M.A. Vega, Human CD36 is a high affinity receptor for the native lipoproteins HDL, LDL, and VLDL, J. Lipid Res. 39 (4) (1998) 777–788.
[23] U. Klein, G. Gimpl, F. Fahrenholz, Alteration of the myometrial plasma membrane cholesterol content with beta-cyclodextrin modulates the binding affinity of the oxytocin receptor, Biochemistry 34 (42) (1995) 13784–13793.
[24] A.J. Brown, L. Sun, J.D. Feramisco, M.S. Brown, J.L. Goldstein, Cholesterol addition to ER membranes alters conformation of SCAP, the SREBP escort protein that regulates cholesterol metabolism, Mol. Cell 10 (2) (2002) 237–245.
[25] S. Rodriguez-Acebes, N. Palacios, J.I. Botella-Carretero, N. Olea, L. Crespo, R. Peromingo, D. Gomez-Coronado, M.A. Lasuncion, C. Vazquez, J. Martinez-Botas, Gene expression profiling of subcutaneous adipose tissue in morbid obesity using a focused microarray: distinct expression of cell-cycle- and differentiation-related genes, BMC Med. Genom. 3 (2010) 61.
[26] A.I. Saeed, V. Sharov, J. White, J. Li, W. Liang, N. Bhagabati, J. Braisted, M. Klapa, T. Currier, M. Thiagarajan, A. Sturn, M. Snuffin, A. Rezantsev, D. Popov, A. Ryltsov, E. Kostukovich, I. Borisovsky, Z. Liu, A. Vinsavich, V. Trush, J. Quackenbush, TM4: a free, open-source system for microarray data management and analysis, BioTechniques 34 (2) (2003) 374–378.
[27] B. Mlecnik, M. Scheideler, H. Hackl, J. Hartler, F. Sanchez-Cabo, Z. Trajanoski, PathwayExplorer: web service for visualizing high-throughput expression data on biological pathways, Nucleic Acids Res. 33 (Web Server issue) (2005) W633–W637.
[28] M.W. Pfaffl, A new mathematical model for relative quantification in real-time RT-PCR, Nucleic Acids Res. 29 (9) (2001) e45.
[29] M. Hao, S.X. Lin, O.J. Karylowski, D. Wustner, T.E. McGraw, F.R. Maxfield, Vesicular and non-vesicular sterol transport in living cells. The endocytic recycling compartment is a major sterol storage organelle, J. Biol. Chem. 277 (1) (2002) 609–617.
[30] D. Wustner, A. Herrmann, M. Hao, F.R. Maxfield, Rapid nonvesicular transport of sterol between the plasma membrane domains of polarized hepatic cells, J. Biol. Chem. 277 (33) (2002) 30325–30336.
[31] D. Wustner, M. Mondal, I. Tabas, F.R. Maxfield, Direct observation of rapid internalization and intracellular transport of sterol by macrophage foam cells, Traffic 6 (5) (2005) 396–412.

[32] K.J. Moore, I. Tabas, Macrophages in the pathogenesis of atherosclerosis, Cell 145 (3) (2011) 341–355.
[33] N. Shibata, C.K. Glass, Macrophages, oxysterols and atherosclerosis, Circ. J. 74 (10) (2010) 2045–2051.
[34] I. Tabas, Cholesterol and phospholipid metabolism in macrophages, Biochim. Biophys. Acta 1529 (2000) 164–174.
[35] N. Tamehiro, Y. Shigemoto-Mogami, T. Kakeya, K. Okuhira, K. Suzuki, R. Sato, T. Nagao, T. Nishimaki-Mogami, Sterol regulatory element-binding protein-2- and liver X receptor-driven dual promoter regulation of hepatic ABC transporter A1 gene expression: mechanism underlying the unique response to cellular cholesterol status, J. Biol. Chem. 282 (29) (2007) 21090–21099.
[36] K.J. Rayner, Y. Suarez, A. Davalos, S. Parathath, M.L. Fitzgerald, N. Tamehiro, E. A. Fisher, K.J. Moore, C. Fernandez-Hernando, MiR-33 contributes to the regulation of cholesterol homeostasis, Science 328 (5985) (2010) 1570–1573.
[37] T.J. Berrodin, Q. Shen, E.M. Quinet, M.R. Yudt, L.P. Freedman, S. Nagpal, Identification of 5alpha, 6alpha-epoxycholesterol as a novel modulator of liver X receptor activity, Mol. Pharmacol. 78 (6) (2010) 1046–1058.
[38] G. Segala, P. de Medina, L. Iuliano, C. Zerbinati, M.R. Paillasse, E. Noguer, F. Dalenc, B. Payre, V.C. Jordan, M. Record, S. Silvente-Poirot, M. Poirot, 5,6-Epoxy-cholesterols contribute to the anticancer pharmacology of tamoxifen in breast cancer cells, Biochem. Pharmacol. 86 (1) (2013) 175–189.
[39] N. Hamada, Y. Ogawa, T. Saibara, Y. Murata, S. Kariya, A. Nishioka, M. Terashima, T. Inomata, S. Yoshida, Toremifene-induced fatty liver and NASH in breast cancer patients with breast-conservation treatment, Int. J. Oncol. 17 (6) (2000) 1119–1123.
[40] M. Matsumura, K. Tashiro, A. Miura, T. Tajima, I. Kinoshita, E. Kojima, A. Yoshizawa, [A case of non-alcoholic fatty liver disease (NAFLD) aggravated after treatment with raloxifene], Nihon Shokakibyo Gakkai Zasshi 108 (12) (2011) 2036–2041.
[41] Y. Hozumi, M. Kawano, M. Miyata, Severe hypertriglyceridemia caused by tamoxifen-treatment after breast cancer surgery, Endocr. J. 44 (5) (1997) 745–749.
[42] F.J. Field, E. Born, S. Murthy, S.N. Mathur, Regulation of sterol regulatory element-binding proteins in hamster intestine by changes in cholesterol flux, J. Biol. Chem. 276 (20) (2001) 17576–17583.
[43] J.J. Repa, G. Liang, J. Ou, Y. Bashmakov, J.M. Lobaccaro, I. Shimomura, B. Shan, M.S. Brown, J.L. Goldstein, D.J. Mangelsdorf, Regulation of mouse sterol regulatory element-binding protein-1c gene (SREBP-1c) by oxysterol receptors, LXRalpha and LXRbeta, Genes Dev. 14 (22) (2000) 2819–2830.
[44] S. Rong, V.A. Cortes, S. Rashid, N.N. Anderson, J.G. McDonald, G. Liang, Y. A. Moon, R.E. Hammer, J.D. Horton, Expression of SREBP-1c requires SREBP-2-mediated generation of a sterol ligand for LXR in livers of mice, eLife 6 (2017) e25015.
[45] S.J. Ellison-Zelski, N.M. Solodin, E.T. Alarid, Repression of ESR1 through actions of estrogen receptor alpha and Sin3A at the proximal promoter, Mol. Cell Biol. 29 (18) (2009) 4949–4958.
[46] M.R. Bratton, B.N. Duong, S. Elliott, C.B. Weldon, B.S. Beckman, J.A. McLachlan, M.E. Burow, Regulation of ERalpha-mediated transcription of Bcl-2 by PI3K-AKT crosstalk: implications for breast cancer cell survival, Int. J. Oncol. 37 (3) (2010) 541–550.
[47] J.C. Bruning, P. Lingohr, J. Gillette, B. Hanstein, H. Avci, W. Krone, D. Muller-Wieland, J. Kotzka, Estrogen receptor-alpha and Sp1 interact in the induction of the low density lipoprotein-receptor, J. Steroid Biochem. Mol. Biol. 86 (2) (2003) 113–121.
[48] S.D. Goldman, R.S. Funk, R.A. Rajewski, J.P. Krise, Mechanisms of amine accumulation in, and egress from, lysosomes, Bioanalysis 1 (8) (2009) 1445–1459.
[49] C.L. Andrew, A.R. Klemm, J.B. Lloyd, Lysosome membrane permeability to amines, Biochim. Biophys. Acta 1330 (1) (1997) 71–82.
[50] L. Liscum, J.R. Faust, The intracellular transport of low density lipoprotein-derived cholesterol is inhibited in Chinese hamster ovary cells cultured with 3-beta-[2-(diethylamino)ethoxy]androst-5-en-17-one, J. Biol. Chem. 264 (20) (1989) 11796–11806.
[51] C.J. Shoemaker, K.L. Schornberg, S.E. Delos, C. Scully, H. Pajouhesh, G.G. Olinger, L.M. Johansen, J.M. White, Multiple cationic amphiphiles induce a Niemann-Pick C phenotype and inhibit Ebola virus entry and infection, PLoS One 8 (2) (2013) 56265.
[52] I. Kristiana, L.J. Sharpe, V.S. Catts, L.H. Lutze-Mann, A.J. Brown, Antipsychotic drugs upregulate lipogenic gene expression by disrupting intracellular trafficking of lipoprotein-derived cholesterol, Pharmacogenomics J. 10 (5) (2010) 396–407.
[53] A. Canfran-Duque, M.E. Casado, O. Pastor, J. Sanchez-Wandelmer, G. de la Pena, M. Lerma, P. Mariscal, F. Bracher, M.A. Lasuncion, R. Busto, Atypical antipsychotics alter cholesterol and fatty acid metabolism in vitro, J. Lipid Res. 54 (2) (2013) 310–324.
[54] J. Sanchez-Wandelmer, A. Davalos, G. de la Pena, S. Cano, M. Giera, A. Canfran-Duque, F. Bracher, A. Martin-Hidalgo, C. Fernandez-Hernando, M.A. Lasuncion, R. Busto, Haloperidol disrupts lipid rafts and impairs insulin signaling in SH-SY5Y cells, Neuroscience 167 (1) (2010) 143–153.
[55] J. Ferno, M.B. Raeder, A.O. Vik-Mo, S. Skrede, M. Glambek, K.J. Tronstad, H. Breilid, R. Lovlie, R.K. Berge, C. Stansberg, V.M. Steen, Antipsychotic drugs activate SREBP-regulated expression of lipid biosynthetic genes in cultured human glioma cells: a novel mechanism of action? Pharmacogenomics J. 5 (5) (2005) 298–304.
[56] M.B. Raeder, J. Ferno, M. Glambek, C. Stansberg, V.M. Steen, Antidepressant drugs activate SREBP and up-regulate cholesterol and fatty acid biosynthesis in human glial cells, Neurosci. Lett. 395 (3) (2006) 185–190.
[57] M.B. Raeder, J. Ferno, A.O. Vik-Mo, V.M. Steen, SREBP activation by antipsychotic- and antidepressant-drugs in cultured human liver cells: relevance for metabolic side-effects? Mol. Cell Biochem. 289 (1–2) (2006) 167–173.
[58] L.M. Johansen, J.M. Brannan, S.E. Delos, C.J. Shoemaker, A. Stossel, C. Lear, B. G. Hoffstrom, L.E. Dewald, K.L. Schornberg, C. Scully, J. Lehar, L.E. Hensley, J. M. White, G.G. Olinger, FDA-approved selective estrogen receptor modulators inhibit Ebola virus infection, Sci. Transl. Med. 5 (190) (2013) 190.
[59] Y. Zhao, J. Ren, K. Harlos, D.M. Jones, A. Zeltina, T.A. Bowden, S. Padilla-Parra, E. E. Fry, D.I. Stuart, Toremifene interacts with and destabilizes the Ebola virus glycoprotein, Nature 535 (7610) (2016) 169–172.
[60] H. Fan, X. Du, J. Zhang, H. Zheng, X. Lu, Q. Wu, H. Li, H. Wang, Y. Shi, G. Gao, Z. Zhou, D.X. Tan, X. Li, Selective inhibition of Ebola entry with selective estrogen receptor modulators by disrupting the endolysosomal calcium, Sci. Rep. 7 (2017) 41226.