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Up-regulation of low-density lipoprotein receptor in human hepatocytes is induced by sequestration of free cholesterol in the endosomal/lysosomal compartment

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Abstract

Up-regulation of low-density lipoprotein receptor (LDLr) is a key mechanism to control elevated plasma LDL-cholesterol levels. In the present paper, we compare the ability of four distinct pharmacological drugs to up-regulate LDLr expression in human hepatocytes. HepG2 cells were treated with the steroidal analog GW707, the oxidosqualene cyclase inhibitor U18666A, the 3β-hydroxysterol Δ^7-reductase inhibitor AY-9944 and the vacuolar-type ATPase inhibitor bafilomycin A1. We found that the four compounds induced sequestration of free cholesterol in the endosomal/lysosomal compartment leading to a positive filipin staining pattern and a complete inhibition of cholesteryl ester synthesis. As a consequence of the sequestration of cholesterol, the expression and the activity of LDLr were strongly induced resulting from a transcriptional effect which was measured by a reporter gene assay. These effects were fully abolished when an exogenous water soluble cholesterol analog was added to the cells. These findings have led to the identification of a common mechanism to up-regulate LDLr expression in human hepatocytes and may represent an interesting alternative approach to identify new hypolipidemic drugs.

Keywords: LDL receptor up-regulation; U18666; AY-9944; GW707; Bafilomycin A1; Lysosome

1. Introduction

Cholesterol homeostasis in the cell is regulated by a complex set of mechanisms that include cholesterol biosynthesis, hydrolysis of cholesteryl esters from lipoproteins internalized into lysosomes, transport to intracellular organelles such as the endoplasmic reticulum (ER) or the mitochondria for esterification into cholesteryl ester or conversion into bile acids [1,2]. Two proteins have been well characterized to play a pivotal role to maintain cholesterol homeostasis, the 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase and the low-density lipoprotein (LDL) receptor (LDLr). HMG-CoA reductase is the rate-limiting enzyme involved in cholesterol biosynthesis and the HMG-CoA reductase inhibitors named statins represent the most widely prescribed drugs for the treatment of hypercholesterolemia [3]. The LDL receptor regulates the catabolism of LDL by the liver by promoting the clearance of atherogenic LDL particles [4]. Both HMG-CoA reductase and LDLr expression is predominantly regulated at the transcriptional level through a negative-feedback mechanism by the intracellular cholesterol pool. This regulation involves a family of membrane-bound transcription factors called sterol-responsive element binding proteins (SREBPs) as well as two recently identified proteins called SREBP cleavage-activating protein (SCAP) and Insig [5–8]. This sterol-mediated regulation of LDLr represents the main mechanism by which the statins exert their hypocholesterolemic effect in humans. However, despite the major success of this class of drugs, some limitations exist due to some side effects and intolerance in some patients. Thus, the discovery of new drugs able to up-regulate the expression of LDLr through a new mechanism of action may represent a

Abbreviations: LDL, low-density lipoprotein; LDLr, LDL receptor; HMG-CoA, 3-hydroxy-3-methylglutaryl coenzyme A; ER, endoplasmic reticulum; SREBP, sterol-responsive element binding protein; SCAP, SREBP cleavage-activating protein; NPC, Niemann-Pick C; ACAT, acyl-coenzymeA-cholesterol acyltransferase; Dil LDL, 3-3′-dioctadecylindocarbocyanine LDL; CAD, cationic amphiphilic drug

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therapeutic alternative to the use of statins. Recently, a new class of potential cholesterol lowering drugs was described that induced a very potent reduction of LDL cholesterol and triglycerides in fat-fed hamsters. In vitro, these compounds clearly act by activating the SCAP/SREBP pathway leading to up-regulation of LDLr expression. Despite convincing evidences that the compounds could bind to SCAP, the precise mechanism whereby they activate the SCAP/SREBP pathway required further studies [9]. Recently, it was demonstrated that the steroidal LDLr up-regulator GW707 was able to disrupt the intracellular cholesterol trafficking in fibroblasts [10], in a similar way that the amphiphilic compound 3-β-[2-(diethylamino)ethoxy]androst-5-en-17-one (U18666A), which is known to inhibit cholesterol synthesis by inhibiting the oxido-squalene cyclase an enzyme present in the cholesterol biosynthesis pathway [11]. U18666 has been described to interfere with the intracellular sterol trafficking by accumulating lysosomal free cholesterol in fibroblasts and Chinese Hamster Ovary cells [12,13]. This cholesterol trafficking defect mimics the cellular lesions observed in fibroblasts from patient affected by the Niemann-Pick type C (NPC) disease [14–16] as well as in the Smith–Lemli–Opitz syndrome fibroblasts [17] or in sphingolipid storage disease fibroblasts [18]. NPC disease is an autosomal disorder caused by mutations in the NPC 1 and 2 proteins which are involved in the cholesterol trafficking from the lysosomes to other cell organelles [19]. Smith–Lemli–Opitz syndrome is another autosomal disorder in which the primary defect is the de novo cholesterol synthesis due to mutation in the 3β-hydroxysterol Δ^7-reductase, the last enzyme in the cholesterol biosynthesis pathway [20]. Sphingolipid storage diseases are disorders that result from defective lysosomal hydrolase leading to accumulation of endogenous lipids in the lysosomes in many different cell types [21]. In some of these metabolic disorders, perturbation of the intracellular transport of LDL-derived cholesterol has been demonstrated in cultured fibroblasts following sequestration of free cholesterol in a late endosomal/lysosomal compartment.

In order to better characterize the mechanism responsible for the up-regulation of LDL receptor, we compared the effects of four unrelated compounds on the sterol trafficking and LDLr expression in the hepatocyte, the major cell involved in the clearance of plasma LDL cholesterol through the LDLr. We decided to use the human hepatoma HepG2 cells as a relevant cell line, as it was previously demonstrated in these cells both sterol-dependent [9,22] and sterol-independent [23,24] LDLr regulation. We selected the LDLr up-regulator GW707 [9], the NPC inducer U18666 [11–13,25], a 3β-hydroxysterol Δ^7-reductase inhibitor AY-9944 [2] and bafilomycin A1, a specific vacuolar-type ATPase inhibitor which has been described to block cholesterol trafficking in macrophages [26].

We found that the four compounds induce the sequestration of free cholesterol thus preventing the delivery of the sterol to the endoplasmic reticulum and its subsequent esterification. Accordingly, all four compounds, by depleting the ER of cholesterol, enhanced the expression of the LDLr. This mechanism may represent an interesting alternative to up-regulate the expression of LDLr in the hepatocytes.

2. Materials and methods

2.1. Materials

GW707, U18666A and acyl-coenzymeA-cholesterol acyltransferase (ACAT) inhibitor 447C88 [27] were synthetized by GlaxoSmithKline. AY-9944, bafilomycin A1, concanamycin A, 25-hydroxy cholesterol and filipin were purchased from Sigma. [^{14}C] oleate and [^{14}C] mevalonolactone were purchased from Amersham. Basal Medium Eagle (BME) medium, RPMI1640 medium, penicillin, streptomycin and FCS were obtained from Gibco. Fluorescent 3-3′-dioctadecylindocarbocyanine LDL (Dil LDL) were purchased from Biomedical Technologies. Lysotracker Red DND-99 was purchased from Molecular Probes.

2.2. Cell line

HepG2 cells were obtained from ATCC. A stable clone was isolated from HepG2 cells transfected with the LDLr promoter coupled to the firefly luciferase reporter gene as indicated in [9]. Stably transfected HepG2 cells were maintained in a humidified incubator (5% CO_2) at 37 °C in BME medium containing 10% fetal calf serum, 100 U/ml penicillin, 100 U/ml streptomycin.

2.3. Filipin staining

HepG2 cells were seeded in 8-well Biocoat plates and incubated for 24 h with vehicle or compounds in RPMI 1640 medium supplemented with 2% lipoprotein-deficient serum and 10 μg/ml human LDL cholesterol. At the end of the incubation period, cells were fixed with 4% paraformaldehyde for 30 min, washed three times with phosphate buffer saline (PBS), incubated 20 min with 1.5 mg glycine in PBS and then stained for 45 min with 50 ng/ml filipin in PBS. Filipin fluorescence was detected by fluorescent microscopy using Axioplan-2 Zeiss (Iena, Germany) microscope using 360/40 nm excitation and 460/50 emission filters.

2.4. Lipid synthesis and mass

HepG2 cells in 24-well plates were incubated in the presence of vehicle or compounds for 6 h in RPMI 1640 medium supplemented with 2% lipoprotein-deficient serum in the presence of 10 μg/ml human LDL cholesterol and labeled with 1.85 kBq (0.05 μCi) [^{14}C] oleate for 6 h to assess cholesteryl ester and triglyceride synthesis or with 9 kBq (0.5 μCi) [^{14}C] mevalonolactone for 24 h to assess cholesterol synthesis. At the end of the incubation period,

lipids were extracted and separated by thin-layer chromatography as described in [28]. Lipids were identified by using purified standards and the radioactivity associated with each individual lipids was quantified using a Phosphorimager screen (Molecular Dynamics). Lipid mass was determined by staining the plates with phosphomolybdic acid and cholesterol was quantified by densitometry.

2.5. Luciferase reporter gene activity

HepG2 cells in 96-well plates were incubated in the presence of vehicle or compounds for 24 h in RPMI 1640 medium supplemented with 2% lipoprotein-deficient serum in the presence of 10 μg/ml human LDL cholesterol. At the end of the incubation period, cell lysates were harvested and firefly luciferase activity (Promega) determined.

Real-time PCR quantification of RNA HepG2 cells in 24-well plates were incubated in the presence of vehicle or compounds for 24 h in RPMI 1640 medium supplemented with 2% lipoprotein-deficient serum in the presence of 10 μg/ml human LDL cholesterol. mRNA were extracted and cDNA produced using RNeasy mini (Qiagen) and Taqman reverse transcription kits (Applied Biosystem). Real-time PCR was performed on Abi-Prism 7009 using Master Mix SYBRgreen kit (Applied Biosystems). Primers were GTTGCTGGCAGAGGAAATGAGAAG and CAAAGGAAGACGAGGAGCACGAT for LDLr, GGCTGAGCTGCCAAATTGGA and CCAGCTTGTGTGTCCTTGGTATTAGA for HMG-CoA reductase and GGGAGCCTGAGAAACGGC and GGGTCGGGAGTGGGTAATTT for ribosomal 18S. Relative abundance of RNA were calculated from the cycle threshold (Ct) using the formula 2^{-Ct} and expressed as arbitrary units.

2.6. LDL uptake assay

HepG2 seeded in Biocoat slides (Becton Dickinson) were incubated in the presence of vehicle or compounds for 24 h in RPMI 1640 medium supplemented with 2% lipoprotein-deficient serum in the presence of 10 μg/ml human LDL cholesterol. 6 μg/ml of fluorescent Dil LDL were added during the last 4 h of incubation. Intracellular fluorscent dye was detected by microscopy using Axioplan-2 Zeiss (Iena, Germany) rhodamine filters with camera (Zeiss, Germany) and quantified by phosphorimager (Molecular Dynamics).

3. Results

3.1. Treatment with GW707, U18666, AY-9944 and bafilomycin A1 disrupt the cholesterol trafficking in HepG2 cells

We explored the potential of the four molecules to perturb the cholesterol trafficking in human hepatocytes. We first compared the ability of the four molecules to affect cholesterol trafficking by using the filipin staining, a specific fluorescent marker of unesterified cholesterol. HepG2 cells incubated for 24 h in lipoprotein deficient medium supplemented with LDL cholesterol and the different drugs (Fig. 1) displayed a positive filipin staining compared to untreated cells. Observation of cells under microscopy clearly revealed a significant number of fluorescent inclusions in the perinuclear region. A similar staining pattern can be observed using the same compounds in human skin fibroblasts (data not shown,) or in human NPC1-expressing CHO cells [10] and closely resemble that observed in fibroblasts from NPC patients, in which the inclusions were reported to be localized exclusively in the endosomal/lysosomal pathway [29,30]. These results suggest that in human hepatocytes, the four compounds induce extensive accumulation of endocytosed cholesterol in this endosomal/lysosomal pathway. To correlate the positive filipin staining observed in HepG2 cells with an accumulation of cholesterol, we then quantified the intracellular free cholesterol mass after lipid extraction. In HepG2 cells incubated with GW707 in similar condition as described in Fig. 1, we observed a significant increase in free cholesterol mass of $35 \pm 2\%$ ($n = 4$) compared to untreated cells. This result is in accordance with that obtained in NPC1-deficient [19] or sphingolipid-treated [18] fibroblasts. The effects of the other compounds could not be observed on cholesterol accumulation due to the additional effects these compounds exert on cholesterol synthesis inhibition (see Fig. 5).

To confirm that sterol trafficking is affected, we examined the effect of the four compounds on cholesteryl ester synthesis. HepG2 cells were incubated with the four compounds and cholesteryl ester synthesis was monitored by incorporation of [^{14}C] oleate. As observed in Table 1, incubation of HepG2 cells with 2 μM GW707, 2 μM U18666, 5 μM AY-9944 or 10 nM bafilomycin A1 inhibit to 85–95% the incorporation of oleate into cholesteryl ester without altering triglyceride synthesis (data not shown), thus confirming that the LDL-derived cholesterol is not

Table 1
Inhibition of cholesteryl ester synthesis induced by GW707, U18666, AY-9944 and bafilomycin A1 in human hepatoma cells incubated for 6 h in absence (without) or in presence (with) of 2 μM 25-hydroxycholesterol

	Without (%)	With 25-hydroxycholesterol (%)
Control	100	100
GW707 2 μM	14	76
U18666 2 μM	5	64
AY-9944 5 μM	7	62
Bafilo 10 nM	11	63
447C88 1 μM	6	5

Cholesteryl ester synthesis was assessed as indicated in Section 2. Values are expressed as % of control and are the mean of duplicates from one representative experiment. The relative amount of radioactivity measured in control ($n = 3$) were respectively 2632 ± 348 a.u. in absence and 4808 ± 680 a.u. in presence of 25-hydroxycholesterol.

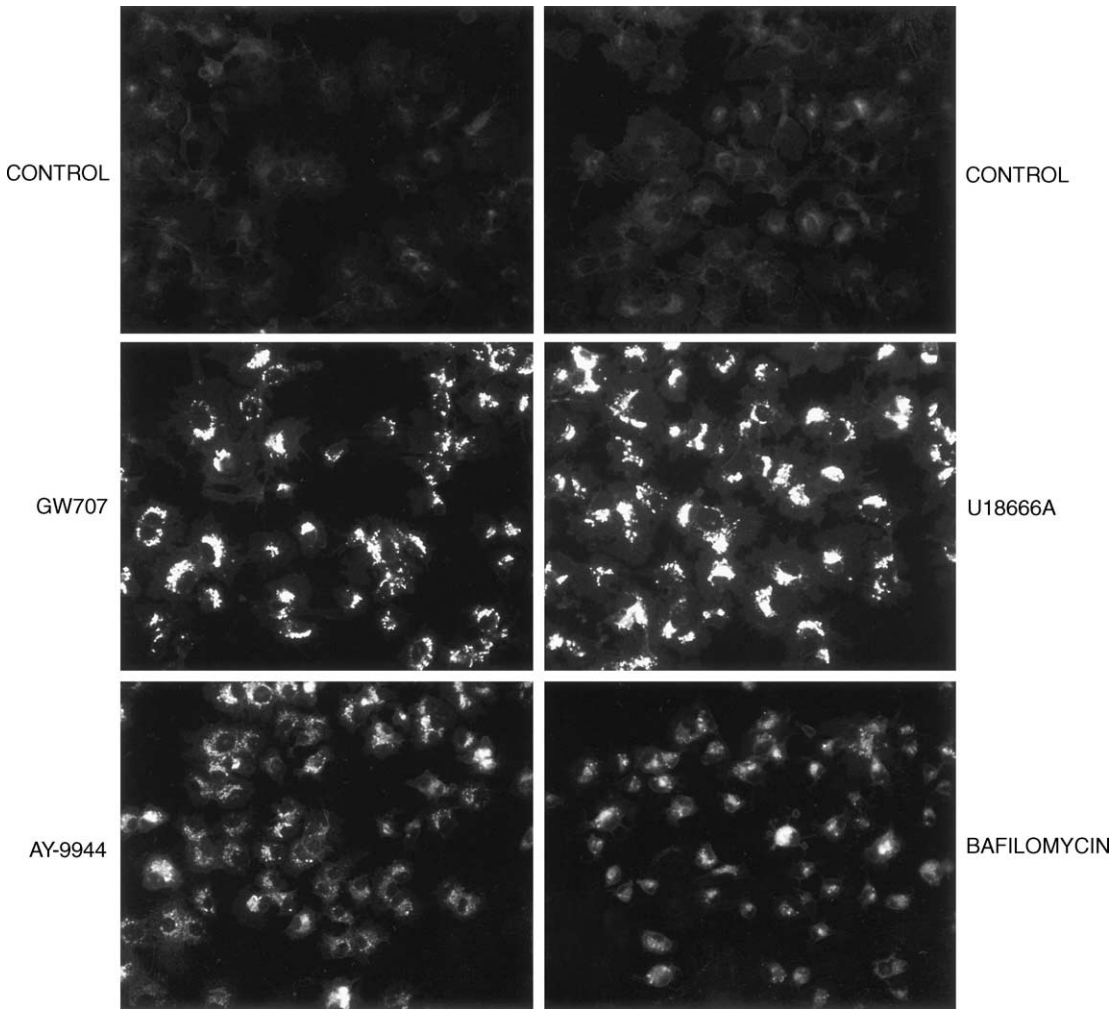

Fig. 1. GW707, U18666A, AY-9944 and bafilomycin A1 induce a positive filipin staining in human hepatoma cells. HepG2 cells were incubated for 24 h without (control) or with 2 μM GW707, 2 μM U18666A, 5 μM AY-9944 or 10 nM bafilomycin A1 and stained for cholesterol using the filipin dye. Cells were examined by fluorescent microscopy as described in Section 2.

available for its conversion into cholesteryl ester. Moreover, when cells were co-incubated with 2 μM of the water soluble cholesterol analog 25-hydroxycholesterol, only a 25–35% reduction of the cholesteryl ester inhibition was observed with the four compounds at the same doses. In contrast, the ACAT inhibitor 447C88 completely inhibits cholesteryl ester synthesis under both conditions, thus suggesting that none of the four molecules were ACAT inhibitors. Dose responses of the four compounds were performed under similar conditions as in Table 1 and the IC50 for inhibition of cholesteryl ester synthesis were respectively 0.02 μM for GW707, 0.2 μM for U18666, 2 μM for AY-9944 and 2 nM for bafilomycin A1 (data not shown).

3.2. Treatment with GW707, U18666, AY-9944 and bafilomycin A1 induce LDLr expression in HepG2 cells

We next studied the effect of the sequestration of free cholesterol obtained with the compounds on the expression of cholesterol-regulated genes such as LDLr. For this purpose, we used a stable HepG2 cell line transfected with a vector containing the human LDLr promoter coupled to the firefly luciferase gene reporter [9].

Stably transfected HepG2 cells were incubated for 24 h with a dose ranging of GW707, U18666, AY-9944 and bafilomycin A1 and luciferase activity was monitored. As observed in Fig. 2, all the four compounds induced a dose-dependent increase in luciferase activity with ED_{50} of respectively, 0.04 μM for GW707, 0.5 μM for U18666, 2.5 μM for AY-9944 and 3 nM for bafilomycin A1 which are very similar to the IC_{50} obtained for inhibition of cholesteryl ester synthesis. Under the same conditions, the ACAT inhibitor 447C88 did not induce any increase in luciferase activity up to 5 μM (data not shown). As shown in the figure, bafilomycin A1 induced a stronger response on luciferase activity compared to the other three compounds. In order to study the competition between the cholesterol and the compounds, HepG2 cells were co-incubated with respectively 10 nM bafilomycin A1, 2 μM GW707, 2 μM U18666 or 5 μM AY-9944 and

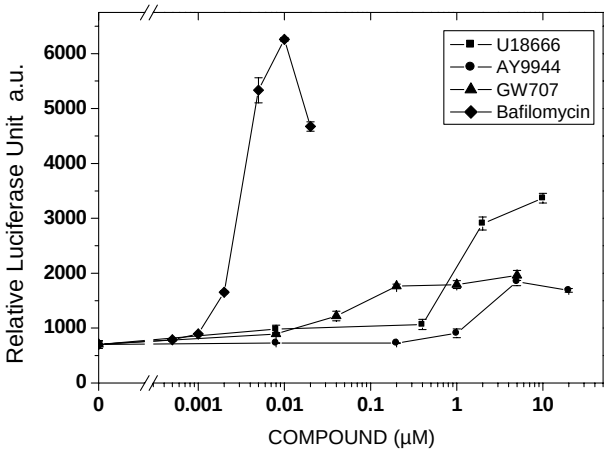

Fig. 2. GW707, U18666A, AY-9944 and bafilomycin A1 activate LDL receptor expression in human hepatoma cells. HepG2 cells were incubated for 24 h without (control) or with increasing doses of compounds and firefly luciferase activity was determined. Values are expressed as relative luciferase units and are the mean ± S.D. of quadruplicates. The relative luciferase units (RLU) under basal conditions (control) were 702 ± 64.

increasing concentration of 25-hydroxycholesterol. As shown in Fig. 3, the hydrosoluble cholesterol analog dose dependently inhibited the luciferase activity induced by the four compounds leading to a complete reversion at 2 μM.

To confirm the effects of the four compounds on the mRNA levels of genes whose expression is regulated by cholesterol, we quantified by realtime RT-PCR the amount of mRNA induced in HepG2 cells incubated for 24 h with GW707, U18666, AY-9944 and bafilomycin A1. Fig. 4 (top) clearly demonstrated a two to three-fold increase in the two cholesterol-regulated genes LDLr and HMGCoA reductase while no effect were observed on the ribosomal 18S mRNA used as a control gene. Similarly to its effect on the LDLr promoter (Fig. 2), bafilomycin A1 induced a stronger effect on gene expression compared to the three other compounds.

To demonstrate that an increase in the mRNA level of LDLr correlates with the enhanced activity of the protein, a functional LDLr receptor assay was set up using the uptake of LDL labeled with the fluorescent dye Dil LDL. The amount of fluorescent dye that accumulates in the cells

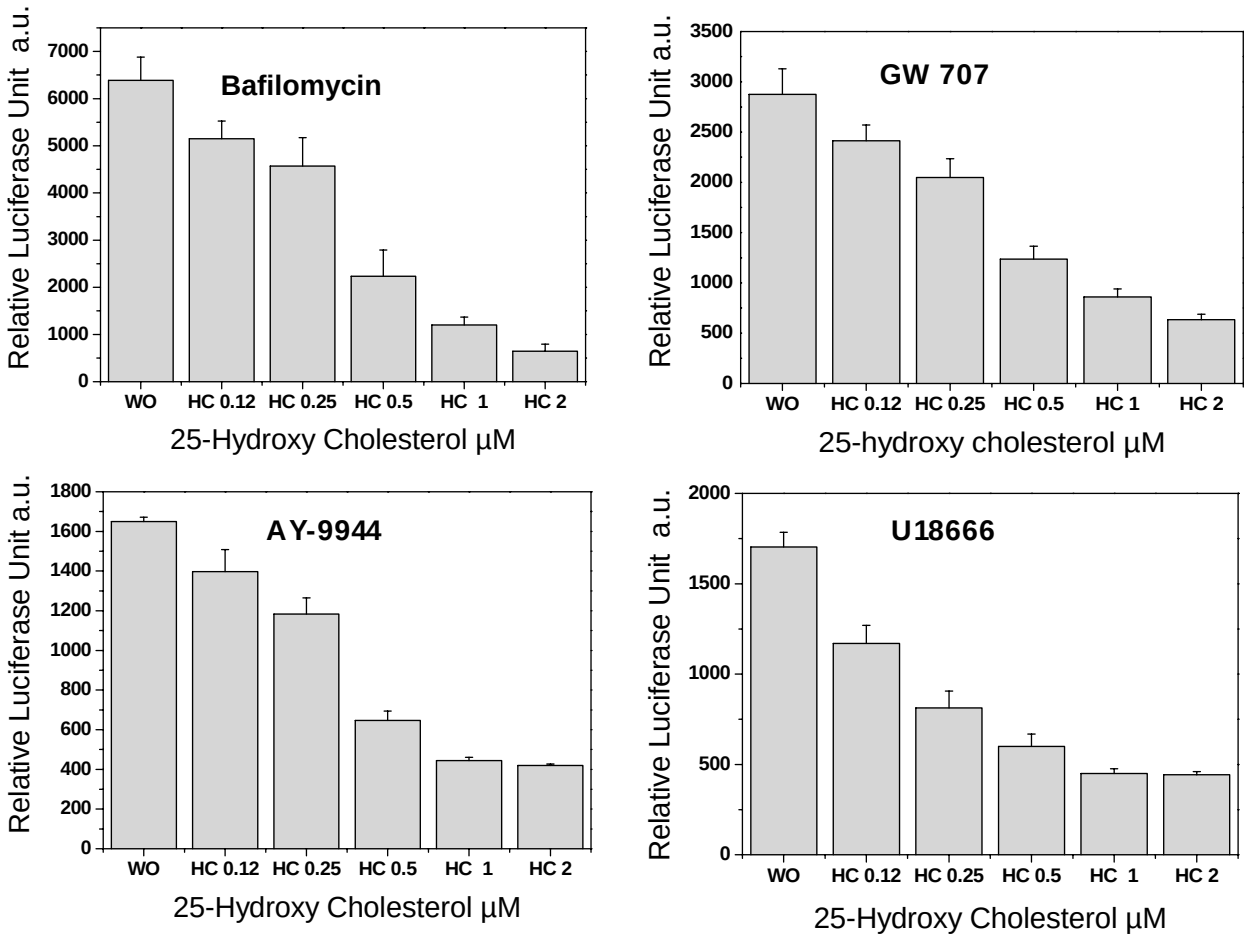

Fig. 3. 25-Hydroxycholesterol reverses the compound-induced LDL receptor expression. HepG2 cells were incubated for 24 h with 10 nM bafilomycin A1, 2 μM GW707, 2 μM U18666A or 5 μM AY-9944 in the absence (WO) or in presence of increasing doses of 25-hydroxycholesterol (HC) from 0.12 to 2 μM. Firefly luciferase activity was determined at the end of the incubation period. Values are expressed as relative luciferase units and are the mean ± S.D. of quadruplicates. The relative luciferase units (RLU) under basal conditions were 470 ± 11 for the study with AY-9944 and U18666 and 1130 ± 79 for the study with bafilomycin and GW707.

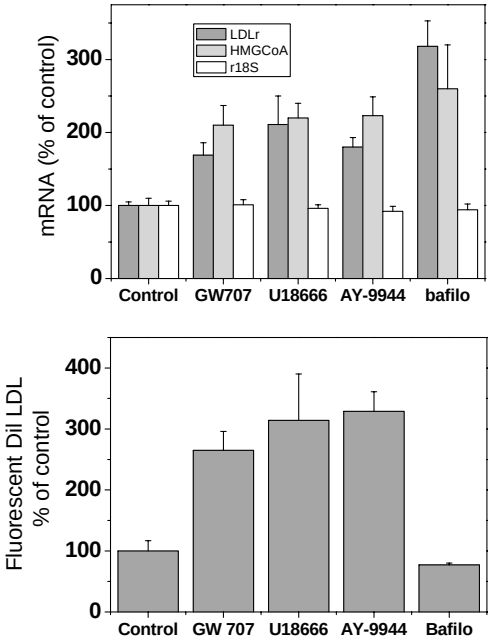

Fig. 4. GW707, U18666A, AY-9944 and bafilomycin A1 induce the expression of a functional LDL receptor in human hepatoma cells. HepG2 cells were treated for 24 h without (control) or with 2 μM GW707 (GW707), 2 μM U18666A (U18666), 5 μM AY-9944 (AY-9944) or 10 nM bafilomycin A1 (bafilo). Top: At the end of the incubation period, mRNA were extracted and quantified by real-time RT-PCR using cDNA for LDL receptor (LDLr), HMG-CoA reductase (HMGCoA) and the ribosomal 18S (r18S). Values are expressed as % of control and are the mean ± S.D. of quadruplicates. The Ct values for control cells were respectively 25.3 ± 0.08 for LDLr, 25.5 ± 0.15 for HMG-CoA reductase and 12.9 ± 0.08 for ribosomal 18S. Bottom: At the end of the incubation period, the uptake of Dil LDL was determined and quantified as indicated in Section 2. Values are expressed as % of control and are the mean ± S.D. of duplicates.

reflects the cell-surface LDLr activity. As shown in Fig. 4 (bottom), treatment of HepG2 cells with 2 μM GW707, 2 μM U18666 and 5 μM AY-9944 led to a 2.5- to 3-fold increase in the quantified fluorescence when compared to control cells, thus indicating a net increase in LDLr activity. With Bafilomycin, we observed a slight decrease in the quantified fluorescence (−23%), due to the fact that the drug probably blocks the recycling of the LDLr at the cell surface as previously demonstrated for the transferrin receptor [31].

3.3. Effects of GW707 and bafilomycin A1, AY-9944 and U18666 on cholesterol synthesis in HepG2 cells

Both AY-9944 and U18666 have been described as potent inhibitors of cholesterol synthesis but act at a different step in the cholesterol synthesis pathway. While AY-9944 inhibits the 3β-hydroxysterol Δ^7-reductase which converts lanosterol into cholesterol [2], U18666 inhibits the oxidosqualene cyclase, an intermediate step in the cholesterol synthesis. Inhibition of this enzyme lead to the generation of 2,3-monoepoxysqualene and 2,3;22,23-diepoxysqualene, the two precursors of the oxydosqualene cyclase [32]. We wanted to know if bafilomycin A1 and GW707 could also affect cholesterol synthesis. To better characterize cholesterol synthesis, we decided to specifically label the cholesterol pathway with mevalonate instead of using acetate which label all the intracellular lipids. HepG2 cells were labeled with [^{14}C]-mevalonolactone and treated respectively with 2 μM GW707, 2 μM U18666, 5 μM AY-9944 or 10 nM bafilomycin. As observed in Fig. 5, the four compounds induced distinct profiles on [^{14}C] mevalonolactone-labeled lipids and some of the minor bands observed on the autoradiograph have not been yet identified. However, quantification of the major band corresponding to cholesterol clearly revealed that U18666 and AY-9944 inhibited cholesterol synthesis to respectively 90 and 71% while GW707 and bafilomycin A1 had no significant effect. As expected, only U18666 but not AY-9944 induced the corresponding formation of 2,3-monoepoxysqualene and 2,3;22,23-diepoxysqualene which characterizes the inhibition of OSC. We can conclude that the four compounds displayed distinct profiles on cholesterol synthesis thus confirming that the inhibition of cholesterol synthesis cannot be responsible for the effects of these four compounds on LDLr up-regulation.

4. Discussion

Up-regulation of LDLr by pharmacological drugs distinct from statins may represent an attractive alternative for therapy in dyslipidemia. In recent years, major clinical trials have shown a strong correlation between the reduction in plasma LDL cholesterol and the reduction in cardiovascular mortality/morbidity [33]. In the present paper, we studied the regulation of LDLr expression in human hepatocytes, induced by pharmacological drugs. Four distinct molecules, with no structural homology, induced a net increase in LDLr expression which correlates with the accumulation of free cholesterol in an intracellular organelle which prevents its esterification. The effect on LDLr is clearly due to an increase in its transcription as demonstrated by gene expression using a reporter gene and by quantification of the mRNA. All four compounds induced accumulation of free cholesterol in lysosomal type structures as demonstrated using filipin staining. The filipin staining pattern obtained in HepG2 cells is identical to that described for U18666 and AY-9944 in fibroblasts [33,34], for U18666 and GW707 in human NPC1-expressing CHO [10] as well as in fibroblasts from NPC patients [29,30] or from Smith–Lemli–Opitz syndrome patients [17]. The positive filipin staining described in all these cell lines correlates perfectly with the inhibition of LDL-stimulated cholesterol esterification due to the fact that cholesterol, the substrate for esterification by ACAT, is not delivered to the endoplasmic reticulum where the reaction occurs. We demonstrated that the four compounds also specifically inhibit cholesteryl ester synthesis in HepG2 cells with a

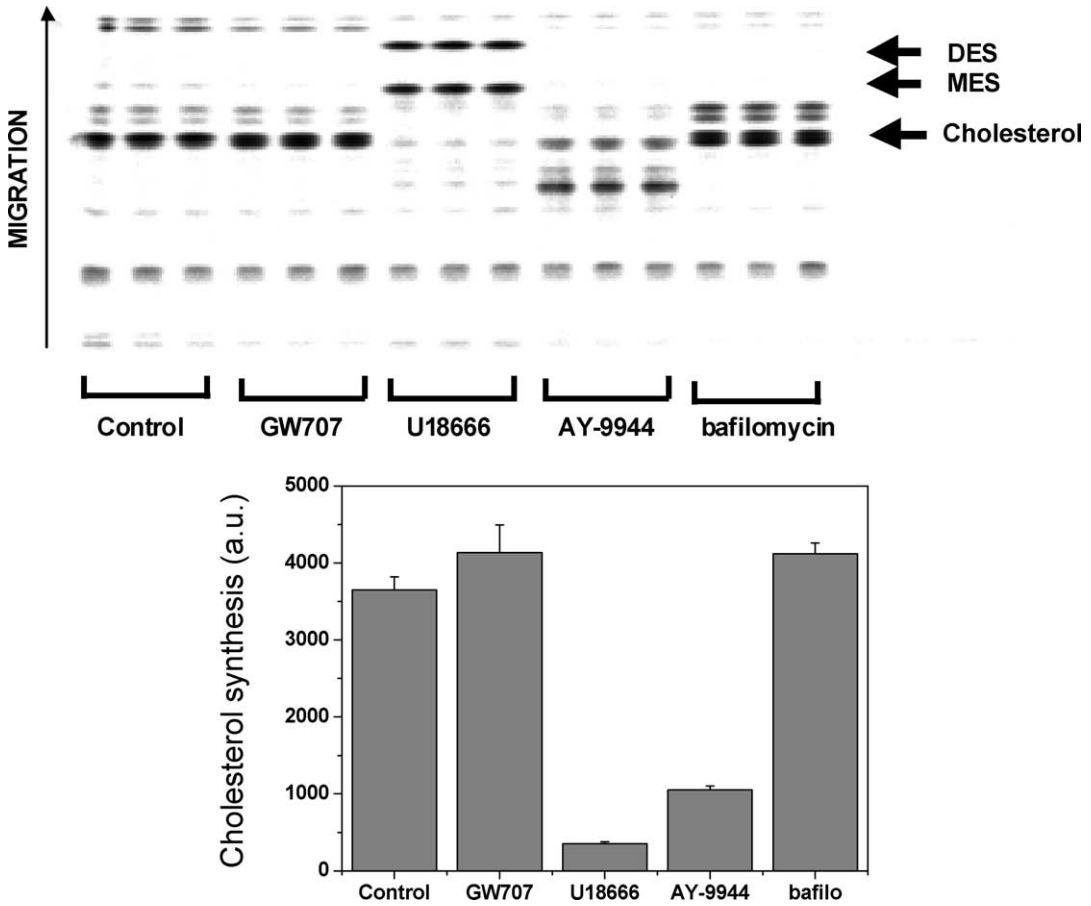

Fig. 5. U18666 and AY-9944 but not GW707 and bafilomycin A1 inhibit cholesterol synthesis in human hepatoma cells. HepG2 cells were treated for 24 h without (control) or with 10 nM bafilomycin A1 (bafilo), 2 μM GW707 (GW707), 2 μM U18666A (U18666) and 5 μM AY-9944 (AY-9944). At the end of the incubation period, cholesterol synthesis was determined as described in Section 2. The top panel shows an autoradiograph of synthetized intracellular lipids separated by thin layer chromatography. The positions of the different lipids have been determined using [^{14}C] (cholesterol) and non-radioactive standards (monoepoxysqualene MES, diepoxysqualene DES). The intensity of the bands corresponding to cholesterol was quantified using a Phosphorimager (bottom panel). Values are the mean ± S.D. of triplicates.

similar potency than that observed to up-regulate LDLr expression. This inhibitory effect on cholesteryl ester synthesis can be overcome by addition to the cells of exogenous cholesterol. When the 25-hydroxycholesterol is added directly to the cell, ACAT is able to use it as substrate in the ER to generate cholesteryl ester. We found that under these conditions, the effects of our four compounds on cholesteryl ester synthesis inhibition was strongly diminished thus confirming that the compounds act at a distal step from the ER.

The direct mechanism responsible for the sequestration of free cholesterol with the four compounds is not totally understood. Only bafilomycin A1 has been clearly demonstrated to specifically inhibit the Vacuolar-type ATPase thus neutralizing the lysosomal proton gradient. The effect of bafilomycin A1 on LDLr expression and sequestration of free cholesterol observed in our study could be attributed to its effect on Vacuolar-type ATPase as another Vacuolar-type ATPase inhibitor, namely concanamycin A, induces exactly the same profile (positive filipin staining, inhibition of cholesteryl ester synthesis, up-regulation of LDLr expression) when added at 50 nM to HepG2 cells. Moreover, we checked that none of the other compounds displayed any effects on vacuolar-type ATPase (data not shown).

We could also postulate that the effects observed in HepG2 cells could be due to the inhibition of cholesterol synthesis as both U18666 and AY-9944 were described as potent cholesterol synthesis inhibitors. We found that neither GW707 nor the Vacuolar-type ATPase inhibitors displayed any inhibitory effects on cholesterol synthesis.

Based on their physicochemical properties, GW707, U18666 and AY-9944 but not bafilomycin A1, can be considered as cationic amphiphilic drugs (CAD). The three molecules contain an hydrophobic ring structure associated with a hydrophilic side chain with a charged cationic amine group. It is well known that some CAD, including drugs such as imipramin or amiodarone can induce a positive filipin staining on cultured fibroblasts [35,36] as well as some excessive accumulation of intracellular phospholipids in vivo, a storage disorder called phospholipidosis [37]. Whether or not the findings observed with these three compounds can be attributed to their CAD property

and are due to the same intracellular mechanism requires further investigations. Recently, it was demonstrated that some cationic amphiphilic drugs such as imipramin but not U18666 were able to induce a conformational change of the SCAP protein thus describing some potential effects of these drugs at the ER level [38]. These recent new properties of some amphiphilic molecules at the level of the ER could explain the data obtained with GW707 on binding to the SCAP protein [9]. In any case, in the present paper, we demonstrated that sequestration of free cholesterol in late endosomal/lysosomal structures induced by distinct mechanisms, translates into a significant increase in LDLr expression in the hepatocyte. This mechanism can lead to a potent reduction of plasma LDL cholesterol in fat-fed hamster as previously demonstrated with another compound [9]. In addition, it was recently proposed that the accumulation of free cholesterol in the ER is directly responsible for the toxicity observed in the macrophages present in the atherosclerotic plaque [39]. Prevention of sterol trafficking from the lysosomes by using NPC1 heterozygous mice confers resistance to lesional necrosis and macrophage apoptosis [40] thus providing in vivo evidences that the mechanism described in this paper, using pharmacological drugs, can represent an interesting novel strategy to stabilize atherosclerotic plaques.

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