PhD Thesis

Effect of antitumoral alkylphospholipids on cholesterol homeostasis

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BIOQUIMICA Y BIOLOGIA

MOLECULAR I

El doctorando Pablo Ríos Marco y los directores de la tesis Josefa Liboria Segovia Parra y María Paz Carrasco Jiménez,

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Abstract

1. Abstract

Alkylphospholipid analogues are a group of antitumoral drugs that, instead of targeting DNA, they are membrane-directed compounds and exert a variety of biological actions. In particular, these agents are promising candidates in cancer treatment, having selective cytotoxicity against many types of tumours, inhibiting the growth of transformed cells. Although alkylphospholipids are currently used in cancer clinic trials, there still exist a high lack of knowledge concerning to their mode of action.

We have worked with two different human tumour cell lines: HepG2 from hepatoblastoma and U-87 MG, from glioblastoma. Cell proliferation and cell cycle progression have been studied in them by flow cytometry and colorimetric assays. All alkylphospholipids tested here are shown to inhibit cell proliferation and cause cell cycle arrest. Interestingly, in the glioblastoma cell line we have obtained data consistent with an induction of different autophagy stages, which are detected by electron microscopy.

Traditionally, phospholipid metabolism was proposed to be the target for alkylphospholipids. However, novel reports from our laboratory and others are linking them with alterations of sterol metabolism in tumoral cells. Given the important roles of cholesterol in cellular functions such as signalling, adhesion and motility, and because its intracellular transport must be strictly regulated for cell growth and membrane biogenesis, in this thesis we execute a study that could prove a relationship between alkylphospholipids and cholesterol homeostasis disruption.

Here we demonstrate that alkylphospholipid-treatment of tumoral cells provoques a clear disruption of the intracellular cholesterol transport. As a consequence, cholesterol biosynthesis and uptake are increased, entailing to an accumulation of cholesterol inside the cell and thereby a disturbance of membrane lipid microdomains. By performing inmunoblotting and fluorescence resonace microscopy, we studied the PI3K/AKT signalling pathway. Remarkably, alkylphospholipids are shown to inhibit this route, being raft-microdomain integrity and cell survival altered as well.

In this work we analyze as well the expression profile of a broad group of genes related with cholesterol anabolism and catabolism demonstrating that there exists an imbalance in cholesterol sensing mechanisms when cells are exposed to alkylphospholipids. Finally, the cholesterol efflux is extensively studied as key part of the cholesterol homeostasis, discovering that alkylphospholipids are able to promote cholesterol efflux from the cell in free-serum condition. In this work we demonstrate that there exists an important influence of the surrounding environment concerning the ability of alkylphospholipids to remove sterols from the plasma membrane.

Therefore the information obtained will be relevant for understanding the interrelationship between all these processes, which is fundamental to our knowledge of tumour response and may facilitate the development of novel therapeutics to improve the cancer treatment.

1. Resumen

Los análogos de alquilfosfolípidos son un grupo de fármacos antitumorales que dirigidos hacia membrana, en lugar de dañar al ADN, ejerciendo una variedad de acciones biológicas. En particular, estos agentes son candidatos prometedores en el tratamiento del cáncer, puesto que presentan citotoxicidad selectiva contra varios tipos de tumores, inhibiendo el crecimiento de células transformadas. A pesar de que los alquilfosfolípidos se utilizan actualmente en ensayos clínicos contra el cáncer, aún existe una gran falta de conocimiento acerca de su modo de acción.

En esta tesis se ha trabajado con dos líneas de células tumorales humanas diferentes: HepG2, de hepatoblastoma y U-87 MG, de glioblastoma. En estas células la proliferación celular y la progresión del ciclo celular han sido estudiadas mediante citometría de flujo y ensayos de colorimetría. Todos los alquilfosfolípidos utilizados en este estudio han mostrado inhibir la proliferación celular y causar detención del ciclo celular. Curiosamente, en la línea celular de glioblastoma, hemos obtenido datos consistentes con una inducción de la autofagia, detectando diferentes etapas de este proceso mediante microscopía electrónica.

Tradicionalmente, se ha propuesto que la diana de estos compuestos es el metabolismo de los fosfolípidos. Sin embargo, trabajos novedosos de nuestro laboratorio y otros están vinculando a los alquilfosfolípidos con alteraciones del metabolismo de esteroles en las células tumorales. Dado el importante papel del colesterol en las funciones celulares, como señalización, adhesión y motilidad y, debido a que su transporte intracelular debe estar estrictamente regulado para permitir el crecimiento celular y la biogénesis de la membrana, en esta tesis se lleva a cabo un amplio estudio que podría mostrar la relación entre el los alquilfosfolípidos y una disrupción de la homeostasis del colesterol intracelular.

Aquí demostramos que el tratamiento de las células tumorales con alquilfosfolípidos provoca una clara interrupción del transporte de colesterol intracelular. Como consecuencia, la biosíntesis de colesterol y su entrada se incrementan, lo que implica a su vez una acumulación de colesterol dentro de la célula y perturbaciones de microdominios de lípidos de membrana. Mediante la realización de microscopía de fluorescencia de resonancia e inmunoblotting, se ha estudiado la vía de señalización PI3K/AKT. Sorprendentemente, los alquilfosfolípidos

inhiben esta ruta, alterando también la integridad de los dominios de membrana raft y la supervivencia celular.

En este trabajo se analiza también el perfil de expresión de un amplio grupo de genes relacionados con el anabolismo y el catabolismo del colesterol demostrando que existe un desequilibrio en los mecanismos de detección intracelular de colesterol cuando las células se exponen a los alquilfosfolípidos. Finalmente, el eflujo de colesterol es extensamente estudiado como parte clave de la homeostasis de colesterol, descubriendo que los alquilfosfolípidos son capaces de promover la salida de colesterol desde la célula en condiciones de ausencia de suero. En este trabajo se demuestra que existe una gran influencia del entorno sobre la capacidad de los alquilfosfolípidos para retirar los esteroles de la membrana plasmática.

Por lo tanto, la información obtenida será relevante para la comprensión de la interrelación de todos estos procesos, lo cual es fundamental para nuestro conocimiento de la respuesta tumoral y puede facilitar el desarrollo de nuevas terapias para mejorar el tratamiento del cáncer.

Introduction

2. Introduction

2.1. Cancer and metabolism

It is estimated that there are over 13 million people diagnosed with cancer until nowadays. Its hardness and difficulty to treat make this disease one of the main points in biomedical research for work in. The damage caused to society by cancer is deep, not only in terms of the patient suffering and their relatives, but the cost of cancer in economic terms.

Cancer treatment has little changed in the last 20 years but significant progress has been made on understanding the molecular mechanisms underlying the process of oncogenic transformation. We have learnt that the cancer cell phenotype is achieved through the reactivation or alteration of existing cellular programmes used for normal cellular homeostasis. These programmes coordinate processes such as cell proliferation, migration, polarity, apoptosis or energy generation (Iaccarino and Martins, 2011). In this sense, differences in energy metabolism between normal and cancer cells had been observed at the beginning of the twentieth century. No cancer cells derive the majority of their energetic needs from ATP through oxidative phosphorylation in the mitochondria. In contrast, tumoral cells exhibit the Warburg effect, that consist in producing ATP even in aerobic conditions through glycolysis, producing lactate from piruvate, a catabolic pathway predominantly less efficient than oxidative phosphorylation. It is known nowadays that one reason for this metabolic switch is that glycolysis provides the majority of the anabolic intermediates required to sustain high rates of cellular proliferation. In addition, tumoral cells cannot use glucose to provide their biosynthetic pathways with a source of reduced nitrogen, but they have solved this problem by increasing their glutamine uptake to feed the Krebs cycle.

Cancers exhibit alterations in several signal transduction pathways governing the survival and proliferation of the cell. For instance, the phosphatidylinositol-3-kinase (PI3K) pathway is one of the most frequently mutated pathways in cancer. This route is known to give rise to tumours that have a significantly elevated glucose uptake and dependency. The PI3K pathway is therefore the subject of several studies and it has been discussed it relation with genes involved in cholesterol and fatty acid biosynthesis (Porstmann et al., 2005). Another well known route related with cell survival is Fas. Fas-mediated apoptosis involves

translocation of Fas and downstream signaling molecules into membrane specific domains, a process that can be pharmacologically modulated. This is of importance in apoptosis-deficient disorders such as cancer and autoimmune diseases (Mollinedo and Gajate, 2006).

On this regard, the development of anti-tumour drugs is one of the most significant challenges that modern medicine has to overcome. The impact of conventional chemotherapeutic agents affects not only tumour tissues, but also rapidly dividing cells of healthy organs (e.g. bone marrow, gastrointestinal epithelial cells and hair follicles). Furthermore, some organs, like the heart, liver and kidney, were also observed to be damaged. One of the major obstacles of the anti-cancer therapy is represented by the multi-drug resistance, whose mechanisms include accelerated drug efflux, drug inactivation, alterations in drug targeting and evasion of apoptosis (Wong and Goodin, 2009). Therefore, it is necessary to develop novel strategies and overcome these severe problems.

2.2. Alkylphospholipids

Alkylphospholipid (APL) derivatives are cytostatic agents that, in contrast to most of the currently used chemotherapeutic drugs, do not target DNA or the cytoskeleton but act at the cell membrane (Van Blitterswijk and Verheij, 2008) and thus they constitute promising candidates for a new approach to cancer chemotherapy (van der Luit et al., 2007; Vink et al., 2007).

In the early 1960s, it was observed that the generation of 2-lysophosphatidylcholine, from phosphatidylcholine (PtdCho) induced the phagocytic activity of peritoneal macrophages in vitro and in vivo (Munder and Modolell, 1973). However, 2-lysophosphatidylcholine is not stable and is inactivated either by the action of acyltransferase generating PtdCho or by lysophospholipase producing glycerophosphocholine; subsequent efforts were made to synthesize metabolically stable phospholipids analogues for clinical research and trials. In these attempts APLs appeared.

Edelfosine represents the first generation of APLs; is an ether lipid (Fig. 1) that accumulates in cell membranes. Hexadecylphosphocholine (HePC), also known as miltefosine, represents the second generation of these compounds and has a simpler structure

that lacks the carbon skeleton of glycerol. Both compounds exert strong cytostatic action in vitro on several tumor cell lines (Van Blitterswijk and Verheij, 2008). In order to improve antitumor activity with reduced side effects, erucylphosphocholine (ErPC) and perifosine emerged. Compared to miltefosine, ErPC contains a longer hydrocarbon chain with a cis double bond and perifosine presents a piperidine moiety instead of the choline head group.

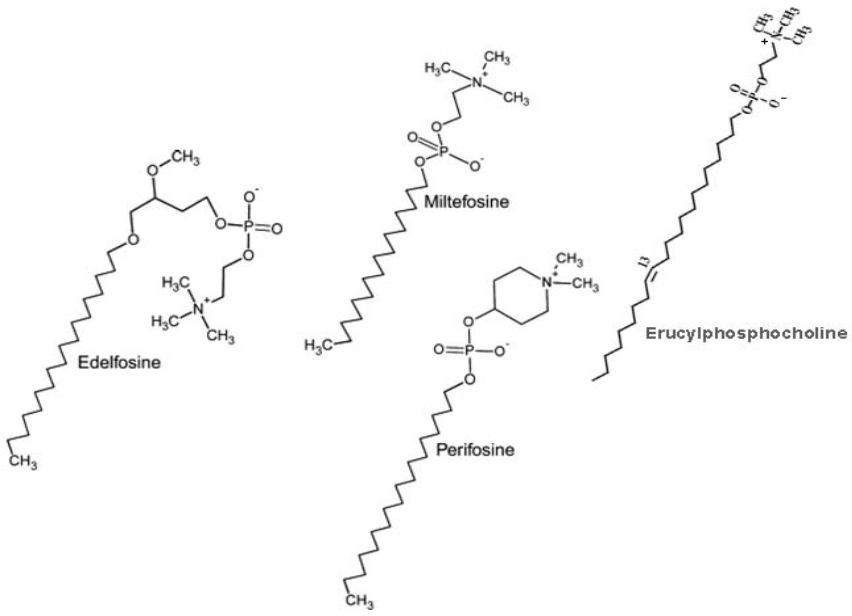

Figure 1. Structure of alkylphospholipids. APLs exhibit a long saturated hidrocarbonated chain, being the ErPC the only one with a double bond. The polar head is mostly derivative from choline with the exception of perifosine.

The inhibition of tumor cell proliferation caused by APLs may be the result not only of inhibition in proliferation but also because of the induction of apoptosis. Some clinical studies have shown promising results; for example, miltefosine may be used for the topical treatment of cutaneous metastases of mammary carcinomas (Clive et al., 1999). This compound exerts antitumor activity too against a broad spectrum of established tumor cell lines and solid tumors (Rybczynska et al., 2001; Jendrossek et al., 2002), in fact some preclinical studies with miltefosine are proving useful information for treating certain cutaneous lymphomas, in which a response of up to 60% effectiveness has been observed

(Dumontet et al., 2006). On the other side, perifosine exerts potent antitumoral activity and is currently undergoing phases II and III of clinical studies for colorectal cancer and for multiple myeloma. Moreover, ErPC and edelfosine have been tested for their anti-tumor activity in clinical phase I and phase II trials for a variety of tumors. Currently, APLs are attractive for use in combination with radiotherapy, since they enhance radiation-induced apoptosis. In this sense, encouraging results have been obtained with APLs in the treatment of leukaemic malignancies (Vink *et al.*, 2007).

A wide variety of molecular mechanisms have been proposed to explain the antitumor activity of distinct membrane-directed APLs, whose action appears to depend on the cell type, the uptake rate into the cell and the compound under study. Due to their chemical structure, APLs are thought to be able to exert their antiproliferative activity through their insertion into both plasma membrane and subcellular membranes, where they accumulate. (Geilen et al., 1994). Until now its mode of action has not been precisely established, although the membrane appears to be the primary site of its activity, most likely due to interference with lipid metabolism and lipid-dependent signal transduction (Barratt et al., 2009). Since many of enzymes involved in lipid metabolism are mainly located in the membrane of the endoplasmic reticulum (ER), this might be a potential target of APLs.

Regarding its specificity, edelfosine for instance was demonstrated to trigger the apoptosis of tumour cells without affecting normal cells (Diomede et al., 1993; Houlihan et al., 1995). Mollinedo and coworkers (1993) proposed that the induction of apoptosis constitutes a relevant step in the cytotoxic activity of edelfosine. Some previous studies from our laboratory demonstrated that nontoxic concentrations of miltefosine exert an antiproliferative effect on cultured HepG2 cells (Jiménez-López et al., 2002). These findings agree with those encountered in MDCK (Wieder et al., 1995), HeLa (Wieder et al., 1993) and other neoplastic cell lines (Boggs et al., 1998), indicating that tumoral cells are sensitive to cytostatic activity of miltefosine. Moreover, we have described (Jiménez-López et al., 2002) that after prolonged treatment with miltefosine, the tumoral cells show a clearly rounded morphology and even became increasingly detached from the plate. In fact, cells exposed to miltefosine for more than 24 h sometime show typical features of apoptosis, such as DNA laddering and caspase-3 activation. Thus, apoptotic cell death induced by miltefosine in the

HepG2 cells appears to involve, at least, an increased caspase-3-like protease activity and genomic DNA fragmentation.

APLs have also shown other biological actions as antiparasitic and antimicrobial effects. So, orally administered miltefosine has been reported to be efficacious against leishmaniasis as a consequence of its interference with the parasite's metabolic pathways (Soto and Soto, 2006; Rakotomanga et al., 2007) inducing apoptosis on it. In fact, HePC has become the first active orally administered agent to prove being effective against visceral and cutaneous leishmaniasis caused by Leishmania donovani and Leishmania panamensis respectively (Pérez-Victoria et al., 2006). It has also recently proved to be useful in the treatment of allergies related to T cells (Bäumer et al., 2009). Obando et al. (2007) analyzed several synthetic derivatives of HePC and edelfosine and concluded that these compounds might also be promising antifungal and antimicrobial drugs. Miltefosine is also toxic in vitro to other protozoan parasites as Trypanosoma cruzi (Saraiva et al., 2002) and some species of Acantamoeba (Seifert et al., 2001).

2.3. Effect of alkylphospholipids on glycerolipid and sphingolipids metabolism

Effects induced by APLs upon a wide range of cellular processes related with glycerolipid metabolism such as the modulation of calcium homeostasis (Henke et al., 1999), alterations of phospholipase C (Berkovic et al., 1996), phospholipase A2 (PLA2) (Berkovic et al., 1997) or phospholipase D activity (Lucas et al., 2001), lipid-signal transduction events (Arthur and Bittman, 2008) and PtdCho metabolism (Berkovic, 1998) has led to several hypotheses being put forward to explain how it works. One of these is that they may arrest tumor cell proliferation by interfering with the biosynthesis of PtdCho.

As depicted in figure 2, in eukaryotes the biosynthesis of PtdCho occurs via two distinct pathways (Vance and Vance, 2004). 1) The main pathway (CDP–choline pathway) consists of three steps: the phosphorylation of choline catalyzed by choline kinase (CK), followed by the transfer of CMP from CTP to choline phosphate, catalyzed by CTP phosphocholine cytidylyltransferase (CT), and finally the transfer of choline phosphate from

CDP-choline to diacylglycerol (DAG), catalyzed by CDP-choline:sn-1,2-diacylglycerol cholinephosphotransferase (CPT) (Vance and Vance, 2004). 2) In the other pathway of PtdCho biosynthesis three successive methylations to convert phosphatidylethanolamine (PtdEtn) to PtdCho are involved. These methylations are catalyzed by PtdEtn N-methyltransferases, enzymes commonly found in hepatic microsomes. PtdCho produced by this route accounts for 20% to 40% of the quantity produced by hepatocytes.

Our research group (Jiménez-López et al., 2002) and others (Geilen et al., 1992; Wieder et al., 1998) have shown that synthesis of PtdCho is inhibited by HePC via CDP-choline pathway. As far as the soluble intermediates in the CDP-choline pathway are concerned, we have found that treatment of human hepatoma cell line HepG2 with this agent produces a significant increase in the label of choline phosphate and a decrease in that of CDP-choline compared to the untreated cells. Thus, the inhibitory effect produced by miltefosine on PtdCho synthesis in these cells seems to be the consequence of an alteration in CT activity. In fact, the exposure of HepG2 cells to HePC caused a dose-dependent increase in cytosolic CT activity and this was accompanied by a concomitant decrease in membrane-bound CT activity in the cell particulate fraction, while the total CT activity was unaltered. Therefore, this APL interferes with PtdCho biosynthesis by impairing the translocation of the rate-limiting enzyme CT from the cytosol, where it is inactive, to membranes, where it expresses activity; that is, it affects only the distribution of CT. Althought, HePC did not inhibit particulate CT activity in vitro, i.e., at the membrane level, it did inhibit cytosolic CT activity in the presence of low amounts of activating PtdCho/oleate liposomes. Thence, miltefosine appears to hinder the insertion of the soluble CT form into lipid vesicles or the membrane to become activated. Interestingly, simultaneous exposure of cells to oleate increased CT activity hereby stimulating PtdCho synthesis so that it drastically reversed the inhibitory effect of miltefosine on PtdCho formation (Jiménez-López et al., 2002).

The reduction in PtdCho biosynthesis was shown not to be due to any alteration in choline uptake by the HepG2 cells, a finding which agrees with that found in MDCK cells (Geilen et al., 1992), but it does go against observations made in neuronal cells (Posse et al., 1995) and KB and Raji cells (Berkovic et al., 1995). In the latter cells, an increase in the degradation of PtdCho was also apparent after miltefosine treatment. The inhibition of PtdCho synthesis in the HepG2 cell line after miltefosine incubation was not related to any

alteration in the degradation rate of PtdCho or its secretion into the culture medium; in addition, treatment with miltefosine altered neither the activity of cytosolic choline kinase nor that of membrane- bound diacylglycerol cholinephosphotransferase (Jiménez-López et al., 2002). It is remarkable that induction of apoptosis by distinct APLs in lymphoma cells occurs through inhibition of CTP-phosphocholine CT after internalization via endocytosis (van der Luit et al., 2007). Since PtdCho is involved in cell-signaling processes, minor alterations in its levels may contribute negatively to cell survival.

Incubation of HepG2 cells with miltefosine was also shown to increase the de novo biosynthesis of triacylglycerol (TAG) and PtdEtn (Jiménez-López et al., 2006). The combined enhancement of TAG and PtdEtn synthesis may be attributed in part to the higher availability of diacylglycerol for glycerolipid biosynthesis when the synthesis of PtdCho is inhibited, as illustrates Figure 2. The synthesis of PtdEtn requires the previous CDP-ethanolamine formation in a two-step process, followed by the transfer of ethanolamine residue to DAG by phosphoethanolamine transferase. Concerning the synthesis of PtdEtn, in our laboratory it has been analyzed the water-soluble intermediates and final product, PtdEtn, of the CDP-ethanolamine pathway and found that HePC causes a modest increase in the incorporation of radiolabeled ethanolamine into CDP-ethanolamine and PtdEtn and a decrease in ethanolamine phosphate, which might be interpreted in terms of a stimulation of CTP:phosphoethanolamine CT activity, the rate-limiting enzyme of this metabolic pathway. Even though these changes might be attributed to miltefosine stimulating the synthesis of PtdEtn in HepG2 cells, the effect is quite slight (only 10%) compared to controls (Jiménez-López et al., 2004).

It has been reported too that miltefosine treatment increases the amount of PtdEtn in the membranes of Leishmania donovani promastigotes (Rakotomanga et al., 2007). Moreover, the ether lipid edelfosine increases the production of CDP-ethanolamine hence enhancing the PtdEtn synthesis in MCF-7 cells (Zhou and Arthur, 1995). Since PtdEtn can be methylated in the ER to obtain PtdCho, it was analyzed this process and observed that miltefosine significantly decreases the microsomal synthesis of PtdCho from PtdEtn by inhibiting PtdEtn N-methyltransferase activity (Jiménez-López et al., 2004). These results constituted the first experimental evidence that the inhibition of the synthesis of PtdCho via CDP-choline by miltefosine is not counterbalanced by any increase in its formation via methylation. On the

contrary, in the presence of miltefosine both pathways seem to contribute jointly to a decrease in the overall synthesis of PtdCho in HepG2 cells, as shown in Figure 2.

With regard to the metabolism of the acidic phospholipid phosphatidylserine (PtdSer), in mammals PtdSer is formed by the action of a base-exchange enzyme, which catalyzes the reversible displacement of the polar head from PtdEtn or PtdCho by serine. The conversion of PtdSer to PtdEtn also occurs via descarboxylation of PtdSer in a reaction catalized by the phosphatidylserine descarboxilase (Fig. 2). The uptake of radioactive serine into PtdSer and other phospholipids is unchanged by miltefosine and neither is the activity of either PtdSer synthase or mitochondrial PtdSer decarboxylase. This demonstrates that the biosynthesis of PtdSer is unaffected by miltefosine in HepG2 cells (Jiménez-López et al., 2004). However, treatment of the human lymphoma Raji cell line with miltefosine also led to an inhibition of PtdCho synthesis via CDP-choline, but enhanced, however, the generation of PtdCho from PtdSer via decarboxylation and methylation processes as a compensatory mechanism (Berkovic et al., 2002), suggesting that the effect of miltefosine on cellular phospholipid metabolism may well differ depending upon the cell type.

It has been showed too that HePC can interact with PLA2, an enzyme in lipid-mediated cell signaling. Snake venom containing PLA2 have been isolated and miltefosine shown ability to inhibit the purified enzyme (Berkovic et al., 1997). Moreover, some experiments in Leishmania Donovani demonstrate that HePC is internalized and promotes activation of PLA2 inside the parasite (Rakotomanga , 2007).

Our research group has previously reported that sphingolipid metabolism is altered after miltefosine treatment. So, HePC produces a marked time-dependent inhibition of sphingomyelin synthesis, using radiolabeled palmitate as exogenous substrate. An accumulation of ceramide was observable after short-term of this agent treatment, which could well be a result of diminished sphingomyelin synthesis (Jiménez-López et al., 2006). These results agree with findings reported in HaCaT cells, showing that the incorporation of choline into sphingomyelin is inhibited by miltefosine concomitantly with an increase in intracellular ceramide levels (Wieder et al., 1998).

Due to the precursor-product relationship, the biosynthesis of sphingomyelin catalyzed by sphingomyelin synthase might be influenced by the inhibition of the PtdCho synthetic

pathway in the presence of miltefosine. Hence, it is worth emphasizing that miltefosine treatment may affect phospholipid homeostasis and hereby the cell membrane functionality by decreasing the synthesis of choline-bearing phospholipids, that is, PtdCho and sphingomyelin (Figure 2), which are key membrane lipid components.

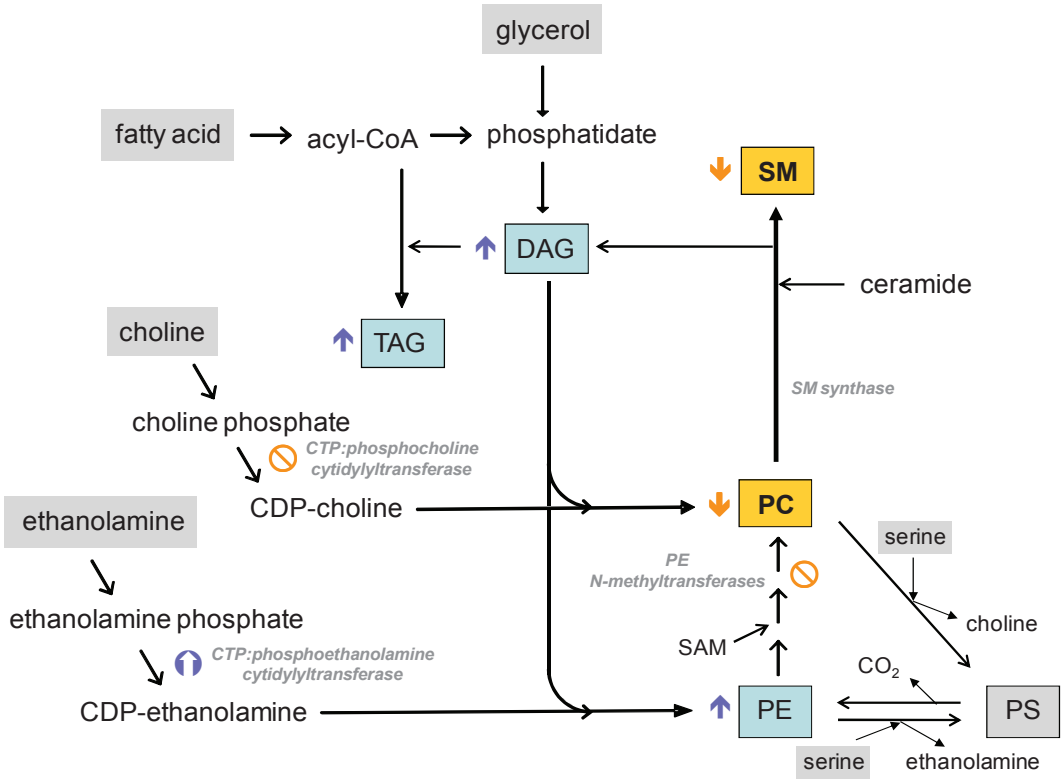

Figure 2. Diagram for the hepatic biosynthetic pathways of phospholipids and triacylglycerol. The postulated mode of action of miltefosine on specific metabolic steps is included. It is noteworthy that miltefosine inhibits the synthesis of both choline-bearing phospholipids, PtdCho (PC) and sphingomyelin (SM). Other abbreviations PtdSer (PS) and PtdEtn (PE).

2.4. Lipid raft domains

Lipid rafts are defined as cholesterol- and sphingolipid-rich domains in biological membranes. They provide specialized lipid environments, which are understood to regulate the organization and function of many membrane proteins (Simons and Gerl, 2010). Rafts are fluctuating assemblies of sphingolipids, cholesterol and proteins (e.g. GPI-proteins) that can be stabilized to coalesce, forming platforms that function in membrane signaling and

trafficking (Coskun and Simons, 2010). Growing evidence of their existence, protein cargo, and regulation is based largely on the study of isolated lipid rafts. Moreover, there is a strict relationship between raft domains and membrane fluidity. In this sense, there are two relevant phases of membranes: liquid disordered (L_d) and liquid ordered (L_o). Sphingolipids, cholesterol and saturated phospholipids contribute to decrease the membrane fluidity and so raft regions are tipically Lo phases (Feigenson, 2006). Lo phases are thicker and less fluid than Ld, so that lipids and proteins diffuse more difficultly.

Regarding raft composition and size, the raft concept has shifted with the realization that the association of components is dynamic and sizes range from small – nanoscale and more stable – to bigger microdomains, which lifetime can vary. The situation is like that of logs in a river: one or several logs can function as a raft for one or more loggers and these can pile up into a raft jam (Simons and Gerl, 2010) (Fig. 3).

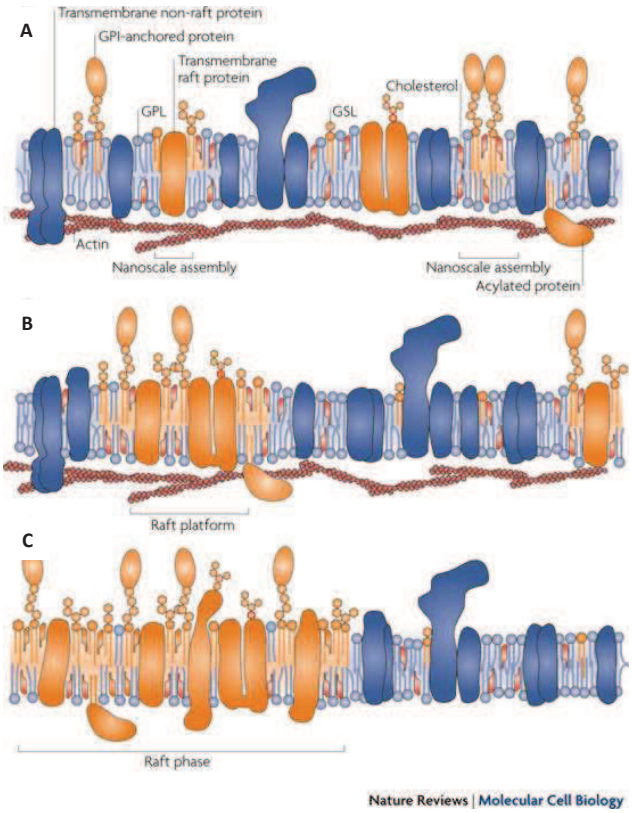

Figure 3. Raft domains formation. Nanoscale assemblies composed of sterols, sphingolipids and different kinds of proteins such as GPI-anchored- , transmembrane-raft- and acylated- proteins. Transmembrane non-raft proteins are excluded from these assemblies (A). In response to external signals or the initiation of membrane trafficking events, raft platforms are formed from fluctuating assemblies through protein–protein lipid–protein, and lipid-lipid interactions. (B). Micrometer-sized raft 'phases' can be induced at equilibrium (C). Abbreviations used: glycosphingolipid (GSL), glycophospholipids (GPL). Figure from Simons and Gerl, 2010.

Although the ganglioside GM1 has been commonly used as raft marker because a fluorescently labeled probe was available, when nanoscale assemblies are analysed, it should not be expected that they are enriched in every marker. Actually, when these assemblies are clustered into raft platforms, there is no obligatory reason why GPI-anchored proteins, GM1 or other raft constituents should be clearly abundant.

Metodology for raft investigation has highly changed in the last years, so that the raft concept has varied from big static structures to a more real and more dynamic model. A key fact ten years ago was the methodology used to define a raft component. The main criteria for raft definition was dependent on its interaction with detergents, so that raft constituents were defined simply as the insoluble residue or "detergent resistant membrane" (DRM) remaining after non-ionic detergent solubilization at 4°C.

The main criticisms for these methods are the use of detergent resistance as a defining factor for raft components (Lichtenberg et al., 2005). Whereas physiologically induced changes in DRM composition can reflect lateral biases in the membrane, detergent solubilization is an inherently artificial method giving different results depending on the concentration and type of detergent, duration of extraction and temperature (Lingwood and Simons, 2007). For this reason, methods that allow raft isolation in absence of detergents are more accurate for raft study composition. In this sense, Macdonald and Pike (2005) have reported a simplified method for raft fractions isolation by the use of free detergent solutions.

Visualizing rafts in cell membranes has always been an important concern that is widely discussed from years ago (Simons and Tommre, 2000). Although microscopically observable probes were used, the variability of the colocalization and sizes seen has induced wonderings about their relevance. Moreover, approaches used to study membrane protein diffusion, such as fluorescence recovery after photobleaching (FRAP) led to mixed results and thus caused skepticism about the raft concept (Kenworthy et al., 2004).

In the past years, an influx of novel methods, such as Fluorescence Resonance Energy Transfer (FRET) and fluorescence polarization anisotropy, revealed that GPI-anchored proteins and other lipid-modified proteins form cholesterol-dependent nanoscale clusters (Meyer et al., 2004; Pinaud et al., 2009). Another tracking methods employed are dual-color total internal reflection fluorescence (TIRF) microscopy and single quantum dot tracking to

study the cholesterol-dependent diffusion behavior of a GPI-anchored protein. These showed that this protein dynamically partitioned into and out of cell surface clusters of the ganglioside GM1 (Pinaud et al., 2009). In spite of some restrictions, data from these methods conclusively support the existence of dynamic nanoscale cholesterol-dependent complexes.

Raft domain assemblies are necessary for activation of some signaling pathways that are related with cell survival or death such as Fas, SRC kinases or AKT/PI3K pathway, being the last one a great point of care. Akt is a serine/threonine protein kinase that plays an important role in cell survival (Benbrook and Masamha, 2011). Akt activation protects cells from apoptotic death and is correlated to the progression of some human cancers. Upstream Akt protein activators are varied receptors, whose activation results in phosphorylation of Akt at Thr308 and Ser473 (Alessi et al., 1997). Remarkably, the interaction between Akt and membrane receptor proteins has been suggested to take place in the lipid rafts of the cell, being shown that Akt is more effectively activated when located in these domains (Gao and Zhang, 2008; Lasserre et al., 2008). In fact, other findings indicate that the phosphorylation of Akt happens faster in raft than in non-raft regions (Gao and Zhang, 2008). Because of all this, the integrity of lipid rafts is required for Akt activity and cell survival so that disruption of lipid raft domains using cyclodextrine destabilizes raft activation platforms of Akt, resulting in impaired Akt phosphorylation at Thr308 and Ser473, diminished Akt phosphorylation, and increased apoptosis in several cell lines (Motoyama et al., 2009; Calay et al., 2010). To determine that the apoptotic response is dependent on cholesterol depletion simvastatin, filipin III and 5-cholesten-5-β-ol have also been used to extract cholesterol, with similar pro-apoptotic effects (Calay et al., 2010).

The results of these studies lead to the conclusion that lipid rafts require proper concentrations of cholesterol and other lipids in order to serve as signaling platforms for Akt activation. In the absence of lipid rafts or proper integrity, Akt activation and cell survival signaling does not take place. In regard to APLs, some authors (Ausili et al., 2008) have shown that, by using phospholipids/SM/cholesterol mixtures, edelfosine alters the phase status of these systems, which are reported to be raft structure models. The same laboratory describes how this APL is accumulated in lipid rafts of cancer cells, altering raft protein and lipid composition. This affects Fas/CD95 death signaling (Gajate and Mollinedo, 2011)

so that it can contribute to apoptosis. All this in mind, raft platforms and key components such as cholesterol may well be a potential pharmacological target in the treatment of cancer.

2.5. Cholesterol metabolism

2.5.1. Cholesterol in cell membranes

Cholesterol is an essential structural component of cell membranes, where it ensures proper membrane permeability, and regulates fluidity over a range of physiological temperatures. It plays a unique role among the lipids in mammalian cells (Maxfield and van Meer, 2010), based partly on its biophysical properties, which allow it to be inserted into or extracted from membranes with relatively easiness. In membranes, cholesterol molecules are intercalated between phospholipids and reduce the movement of their acyl chains, contributing to the maintenance of membrane stability. Thus, it plays a special role in determining the biophysical properties of other lipids in a bilayer. Because of its importance, cells have evolved complex mechanisms to tightly regulate the abundance and distribution of sterols within cells.

Inside the membranes, cholesterol is able to interact with other membrane lipids as well as with specific proteins. Its cyclopentanoperhydrofenanthrene derived structure has unique biophysical properties that increase cohesion and packing of neighbouring lipids. Because of the inflexible sterol structure, cholesterol is preferentially located near lipids containing saturated hydrocarbon chains as these are more rigid and elongated than unsaturated lipids. Cholesterol increases lateral ordering of lipids affecting the biophysical properties of cell membranes, this decreases its fluidity and thus reduces the permeability of polar molecules. On contrary, too much cholesterol is unfavorable as it could slow down the diffusion of membrane proteins. Some models have been suggested to explain cholesterol location within membranes and its interaction with the other lipids (Fig. 4). The simplest interaction shows polar head of phospholipids covering cholesterol polar group; other lipids such as sphingomyelin can collaborate with phospholipids to create bigger association with cholesterol. Some authors have suggested that, depending on cholesterol content, some molecules of cholesterol can be partially exposed to extracellular acceptors. This cholesterol

is termed as "active cholesterol" and it thought to be easily extractable from the membrane (Steck and Lange, 2010).

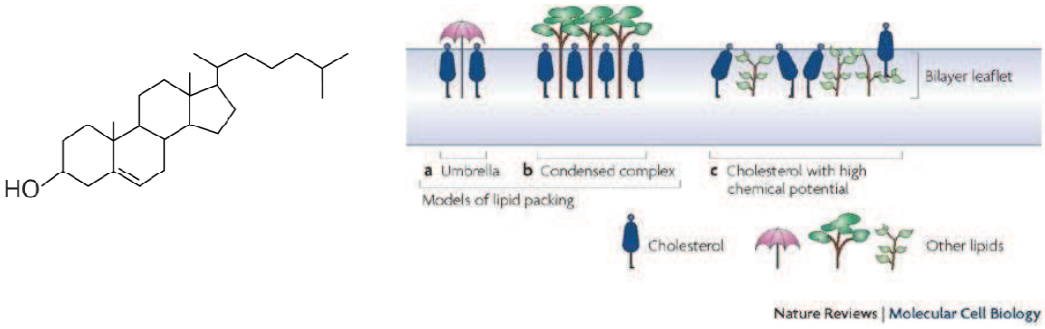

Figure 4. Cholesterol structure and disposition within membranes. Cholesterol structure is a four-ring backbone, with two residues methylated and a radical chain, setting a 27 total number of carbons molecule. It C3 hydroxyl group confers ability to create hydrogen bonds (left). Cholesterol is represented by different models of interaction with other lipids present in membrane (right). The umbrella model (a) shows a simpler interaction, with phospholipids "covering" hydroxyl group of cholesterol with their polar heads. When membrane is enriched in cholesterol more intricate models are suggested (b). Some molecules of cholesterol in membrane can be partially exposed to easier extraction, (c). Taken from Ikonen, 2008.

Cholesterol homeostasis in mammals is regulated by a complex set of mechanisms that include cholesterol biosynthesis, hydrolysis of cholesteryl esters (CE) from internalized lipoproteins, transport of released cholesterol to intracellular organelles such as the ER and cholesterol efflux (Chang et al., 2006). In the liver, many aspects of metabolism of cholesterol are well known, including its synthesis in the ER, its extracellular transport in plasma lipoproteins, its uptake by the low-density lipoprotein receptor (LDLR), and its sterol-level dependent feedback regulation. The pathways involved in cholesterol metabolism are strictly related to its transport and intracellular distribution among subcellular organelles and the plasma membrane (Soccio and Breslow, 2004). However, these pathways and their molecular regulation are still only partially understood.

Our research group has preliminarily examined the effects of the HePC on intracellular cholesterol transport and metabolism and it possibly relevance in maintaining cholesterol

homeostasis. It was shown that treatment of HepG2 and Vero cells with HePC significantly alters cholesterol metabolism and leads to an accumulation of cholesterol in the cell (Jiménez-López et al., 2006). Using radiolabeled substrates we determined the effect of HePC on cholesterol synthesis and found that long-term exposure of HepG2 cells to miltefosine caused a marked increase in cholesterol biosynthesis when acetate, but not mevalonate, was used as the lipogenic precursor. Interestingly, some other experiments confirmed that miltefosine stimulates not only the cholesterogenic pathway but the receptor-mediated uptake of cholesterol in HepG2 cells.

2.5.2. Cholesterol biosynthesis

Cholesterol biosynthesis has been the subject of extensive study and its complex biosynthetic pathway is now well known (Fig. 5). This pathway involves several cytosolic and peroxisomal reactions (Kovacs and Krisans, 2003), converting acetyl-CoA into the intermediate farnesyl diphosphate (FPP). This first stage involves I) conversion of acetyl CoA into acetoacetyl CoA catalyzed by acetoacetyl CoA thiolase (ACAA2), II) conversion of acetoacetyl CoA into 3-hydroxyl-3-methylglutaryl CoA by hydroxyl-3-methylglutaryl CoA synthase (HMGCS1), III) conversion of 3-hydroxy-3-methylglutaryl CoA into mevalonic acid (MVA) by the action of 3-hydroxy-3-methylglutaryl CoA reductase (HMGCR), IV) phosphorilation of MVA into phosphomevalonate by mevalonate kinase (MVK), (V) another phosphorilation of phosphomevalonate into diphosphomevalonate by phosphomevalonate kinase (PMVK) and VI) conversion of diphosphomevalonate into isopentyl diphosphate (Δ3-IPP) by mevalonate diphosphate decarboxylase (MVD). Several of these enzymes have been reported to regulate this process, being essential the HMGCR. This enzyme is located in the ER and is considered to be the rate-limiting enzyme of the cholesterol biosynthetic pathway (Trapani and Pallottin, 2009; Jo and Debose Boyd, 2010), although MVK (Waterham, 2002) and MVD (Rezen et al., 2011) also seem to take part in the control of cholesterogenesis. The enzymatic steps that produce farnesyl diphosphate (FPP) include several branching points that lead towards the synthesis of essential molecules with key roles in different cell processes in addition to cholesterol itself (Fig. 5). Nonsterol isoprenoids, such as heme-A and isoprene, result from the mevalonate pathway (Rezen et al., 2011).

Δ3-IPP, the basic C5 building block, is then added to prenyl diphosphate cosubstrates to form longer chains. Δ3-IPP itself is not reactive enough to initiate the condensation of

higher isoprenoids (Nes, 2011). So, it is first isomerized to the allylic ester Δ2-IPP through rearrangement followed by head to tail condensation of Δ2- and Δ3-IPP to form geranyl diphosphate by geranyl diphosphate synthase. In the second stage, the condensation reaction is repeated by the addition of Δ3-IPP producing the C15 allylic FPP. FPP is a key intermediate in the synthesis of the lipid dolichol and in farnesylation of proteins in which the polyprenyl group serves as a membrane anchor (Bełtowski et al., 2009). A large number of experimental and clinical studies have suggested that inhibition of the production of non-sterol isoprenoids might have a role in human pathology, since they are essential for cell growth, differentiation and apoptosis (Miquel et al., 1996). FPP is also the precursor for coenzyme Q, which is a component of the electron transport chain in mitochondria and participates in aerobic cell respiration to generate energy.

Two molecules of FPP condense tail to tail to the C30 acyclic squalene by the action of squalene synthase (FDFT1). The squalene undergoes oxidation to form S-oxidosqualene via an NADPH-dependent mono-oxygenase reaction catalyzed by squalene epoxidase, and this substrate can be cyclized by an oxidosqualene sterol synthase to yield the steroidal backbone structure presented in lanosterol. In the last stage lanosterol is converted to cholesterol in a multistep process involving many intermediates that are linked to ER (Waterham, 2002). Cholesterol synthesis by microsomal enzymes is fast and extremely efficient to the extent that organization via a multienzymatic complex has been suggested (Gaylor, 2002).

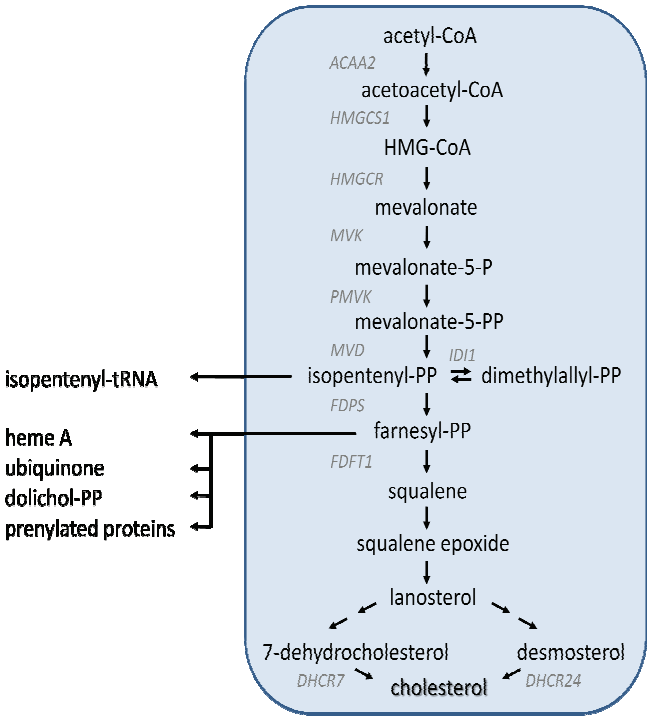

Figure 5: Diagram of the biosynthetic pathway of cholesterol. The product FPP (farnesyl-PP) is utilized in the synthesis of cholesterol, farnesylated and geranylgeranylated proteins, dolichols, coenzyme Q and the isoprenoid moiety of heme. Abbreviations used: acetyl-CoA acyltransferase 2 (ACAA2), acetoacetyl- HMG-CoA synthase (HMGCS1), HMG-CoA reductase (HMGCR), mevalonate kinase (MVK), isopentenyl diphosphate (IPP), phosphomevalonate kinase (PMVK), diphosphomevalonate decarboxylase (MVD), IPP isomerase (IDI1), farnesyl diphosphate synthase (FDPS), squalene synthase (FDFT1) 7-dehydrocholesterol reductase (DHCR7), 24-dehydrocholesterol reductase (DHCR24).

Because free cholesterol is one of the major components of membrane lipids, each cell must balance the internal and external sources while avoiding either a sterol shortage or over-accumulation so as to maintain membrane cholesterol homeostasis. From its site of synthesis, cholesterol needs to be transported to other cell destinations, accumulating mainly in the plasma membrane, where most of cell cholesterol resides (Warnock et al., 1993). On opposite, the cholesterol composition of ER membrane is minimum (lower than 5%) and there exist a highly fine cholesterol sensing mechanism located in this organelle. The smallest changes in cholesterol levels within ER (because of synthesis, arrival or sorting) are able to modulate biosynthetic enzymes as HMGCR by a feedback-control mechanism that is explained in detail later.

Several studies have shown that, in addition to synthesizing cholesterol, mammalian cells also synthesize substantial amounts of intermediates of sterols (Echevarria et al., 1990). In a similar way to cholesterol, these sterols leave the ER and rapidly reach the plasma membrane before moving back to the ER to be processed enzymatically into cholesterol, this movement being essential to the completion of cholesterol biosynthesis (Heino et al., 2000; Field et al., 2007). It has also been shown that when the traffic of cholesterol from the plasma membrane to the ER is disrupted, the circuit for its synthesis is affected, so intermediates of cholesterol biosynthesis accumulate (Metherall et al., 1996; Field et al., 2007).

Moreover, the endocytic pathways plays a key role for cholesterol homeostasis and it is now well established that control of both cholesterol levels and distribution through intracellular traffic contributes to the maintenance of cell cholesterol homeostasis (Soccio and Breslow, 2004). Nevertheless, in contrast to cholesterol's well known metabolism, the routes involved in cholesterol transport and their molecular regulation are still only partially understood. In any case, cholesterol quantities and intracellular distribution are tightly regulated by intracellular transport (Maxfield and Wustner, 2002; Soccio and Breslow, 2004).

2.5.3 Cholesterol uptake and transport

In addition to synthesis, cells obtain cholesterol from the blood via the LDLR and hydrolyze it to free cholesterol in endosomes/lysosomes (Goldstein and Brown, 2009). LDLR, which is present on the plasma membrane of most cells, bind particles that contain ApoB or ApoE proteins, such as chylomicron remnants, very low density lipoprotein (VLDL) and LDL. LDLR complexes are present in clathrin-coated pits on the cell surface, which when bound to LDL-cholesterol via adaptin, are pinched off to form clathrin-coated vesicles inside the cell. This allows LDL-cholesterol to be internalized in a process known as endocytosis and prevents the LDL from merely diffusing around the membrane surface. The vesicles lose their clathrin coats, undergo fusion with other similar vesicles, and form larger vesicles known as early or sorting endosomes. The lower pH in early endosomes promotes the dissociation of LDL from the LDLR. In this compartment cholesteryl esters are hydrolysed by acid lipase to provide unesterified cholesterol for cellular needs. The LDLR and other recycling proteins then localize in the recycling endosomes, from where they return to the plasma membrane after about 10 min to be reused in many more rounds of LDL delivery. Eventually the LDLRs enter the late endosomes en route to being degraded in the lysosomes.

Lysosome membranes are normally cholesterol-poor, which is favourable for sphingolipid digestion (Kolter and Sandhoff, 2005). This suggests that most of the cholesterol normally leaves the endosome membrane before entering lysosomes towards favorable concentration gradient (Fig 6). How this happens has not been clearly stated. The intra-endosomal membranes of multivesicular late endosomes that are enriched in lysobisphosphatidic acid/ bismonoacylglycerophosphate (LBPA/BMP) serve as important regulators of cholesterol transport (Kobayashi et al., 1999).

Abnormalities affecting cholesterol transport and distribution lead to several inherited metabolic illnesses such as Niemann Pick Type C (NPC) disease, characterized by the accumulation of unesterified cholesterol and other lipids in the endosomal/lysosomal compartment (Blanchette-Mackie, 2000). Late endosomes are normally dynamic structures but they become static, enlarged and cholesterol-rich in the NPC cell (Ko et al., 2001). Two independent proteins responsible for this neurodegenerative disorder have been identified; NPC1 is a polytopic membrane protein from late endocytic membranes, whereas NPC2 is a cholesterol-binding soluble protein that also targets the late endocytic organelles. Genetic and phenotypic evidence in mutant mice suggest that both NPC proteins participate in different steps of the same pathway being both necessary for correct running. Both proteins can bind sterol (Xu et al., 2007)) but have also been implicated in sphingolipid binding or mobilization (Malathi et al., 2004). NPC2 is a small soluble protein that functions as a cholesterol transfer protein *in vitro,* favoring acidic pH and the presence of LBPA (Cheruku et al., 2006). By contrast, NPC1 is a glycoprotein that contains 13 membrane-spanning domains (Davies and Ioannou, 2000). A possible mechanism is that NPC2 transfers cholesterol either from intra-endosomal membranes or directly from acid-lipase-catalysed ester hydrolysis to NPC1, participating on its sorting from the endosomes. This could involve NPC1-mediated cholesterol transport across the membrane and sterol egress to cytosolic lipid transfer proteins, or NPC1-regulated membrane transport that removes cholesterol, and possibly sphingolipids (Koivusalo et al., 2007), from late endosomal circuits. Some amphiphilic drugs, such as U18666A, progesterone and imipramine, have been described as interfering with intracellular sterol traffic by accumulating lysosomal unesterified cholesterol in fibroblasts and Chinese Hamster ovary cells (Lange et al., 1998; Mohammadi et al., 2001); the movement of cholesterol from the cell surface to the ER is inhibited by these drugs, which also affect cholesterol transport from the endosomes (Lange, 1994). This defect in cholesterol transport

causes cell damage and mimics the cellular lesions observed in fibroblasts from patients affected by NPC disease, producing an accumulation of unesterified cholesterol and other lipids in the endosomal/lysosomal compartment (Blanchette-Mackie, 2000; Wojtanik and Liscum, 2003). However the precise functions and trafficking itinerary of both NPC1 and NPC2 remain to be clarified.

The contribution of other endocytic routes to cholesterol transport and balance and their interplay with the LDLR route, are yet poorly understood at the molecular level. Cholesterol can move between the ER and the plasma membrane by mechanisms that bypass, in part, the secretory pathway and are probably non-vesicular. Little is known about these mechanisms, but it is likely that they contribute significantly to maintaining the different cholesterol concentration inside the ER and the plasma membrane. On release from the endolysosomal system, cholesterol is delivered to other membranes, such as the plasma membrane, ER, recycling endosomes and mitochondria. Which membrane/organelle serves as the first acceptor for the cholesterol that leaves endosomes has not been determined because of the rapid kinetics by which cholesterol reaches other destinations and insufficient resolution of the available assays (Ikonen, 2008).

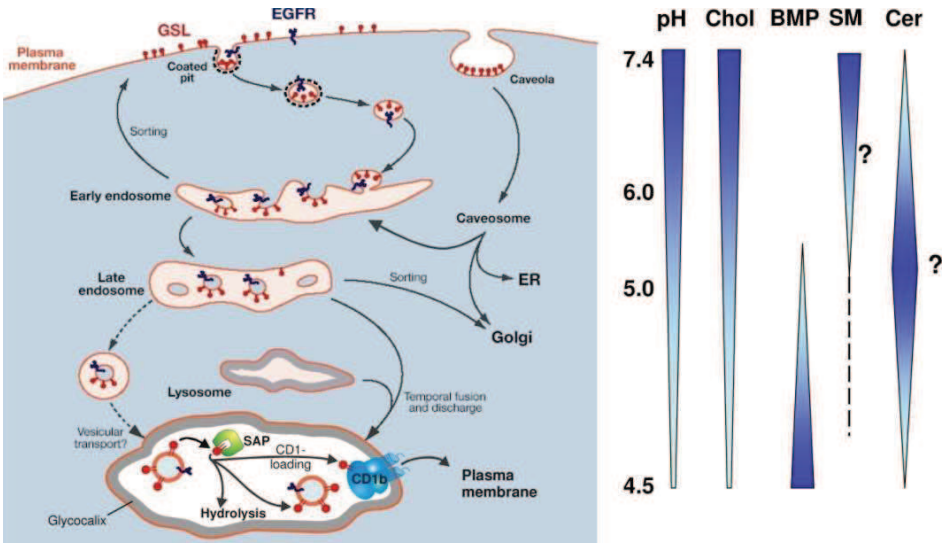

Figure 6. Model of endocytosis and lysosomal digestion of membranes. Glycosphingolipids (GSL) are highlighted on the plasma membrane and on internal membranes, and gradients of pH, cholesterol (Chol), BMP, sphingomyelin (SM; hypothetical), and ceramide (Cer; hypothetical) are shown. Taken from Kolter and Sandhoff, 2010.

Once cholesterol reaches ER it is esterified by acyl-CoA:cholesterol acyltransferase (ACAT) to form cholesteryl esters (CE). Due to the high hydrophobic nature of CE, they cannot insert into membranes; instead of this, they accumulate into lipid droplets, which function as cellular stores of cholesterol. CE formation is an important buffering mechanism for reducing sterol levels when plasma membrane is saturated of free cholesterol. Fatty acid sterol esters are stored too in lipid droplets from the ER. Lipid droplets were considered to be passive fat deposits but they are now emerging as dynamic, regulated organelles (Martin and Parton, 2006). In some cells, such as adipocytes, considerable amounts of unesterified cholesterol are stored in lipid droplets (Prattes et al., 2000). One interesting protein in this respect is caveolin, which associates with both caveolae and lipid droplets (Parton and Simons, 2007). Cholesterol addition was found to induce the trafficking of plasma membrane caveolin to lipid droplets, where caveolin had a role in maintaining unesterified cholesterol levels (Le Lay et al., 2006). There are also some indications of crosstalk between caveolae and lipid droplets in the metabolism of triglycerides (Ost et al., 2005). Moreover, recent data indicate that the ATP-binding cassette (ABC) transporter ABCG1, which facilitates the removal of cellular cholesterol (see below), also regulates triglyceride storage (Buchmann et al., 2007). How cholesterol and triglyceride storage and mobilization in lipid droplets are interlaced are determinant open questions.

As summary, intracellular cholesterol transport it is represented in figure 7. There are three organelles involved in cholesterol trafficking: (1) ER, the major site of synthesis, regulation and esterification of cholesterol, (2) plasma membrane, a prominent storage site for unesterified cholesterol, and (3) endosomes/lysosomes, where lipoprotein-derived cholesterol is released. Endocytosed LDLs are delivered rapidly to lysosomes; the protein/phospholipid coat is degraded and CE are hydrolyzed to cholesterol (Sugii et al., 2003). Most, perhaps all, of this cholesterol is transported directly to the plasma membrane (Lange et al., 1997), which contains approximately 65-90% of the unesterified cholesterol in the cell (Liscum and Munn, 1999). Cholesterol synthesized in the ER, as well as that released in the endosomes/lysosomes by lipoprotein catabolism, moves to the plasma membrane against a steep concentration gradient (Prinz, 2002; Soccio and Breslow et al., 2004) (Fig. 6). Once the capacity of the plasma membrane and other compartments to absorb cholesterol is exceeded, cholesterol is

transported back to the ER, where it is esterified by ACAT. In the ER cholesterol promotes HMGCR proteolysis and inhibits its biosynthesis and uptake by gene expression modulation (explained in more detail later). This distribution of cholesterol between sites of regulation, synthesis, and deposition provides a highly efficient control for cellular cholesterol levels.

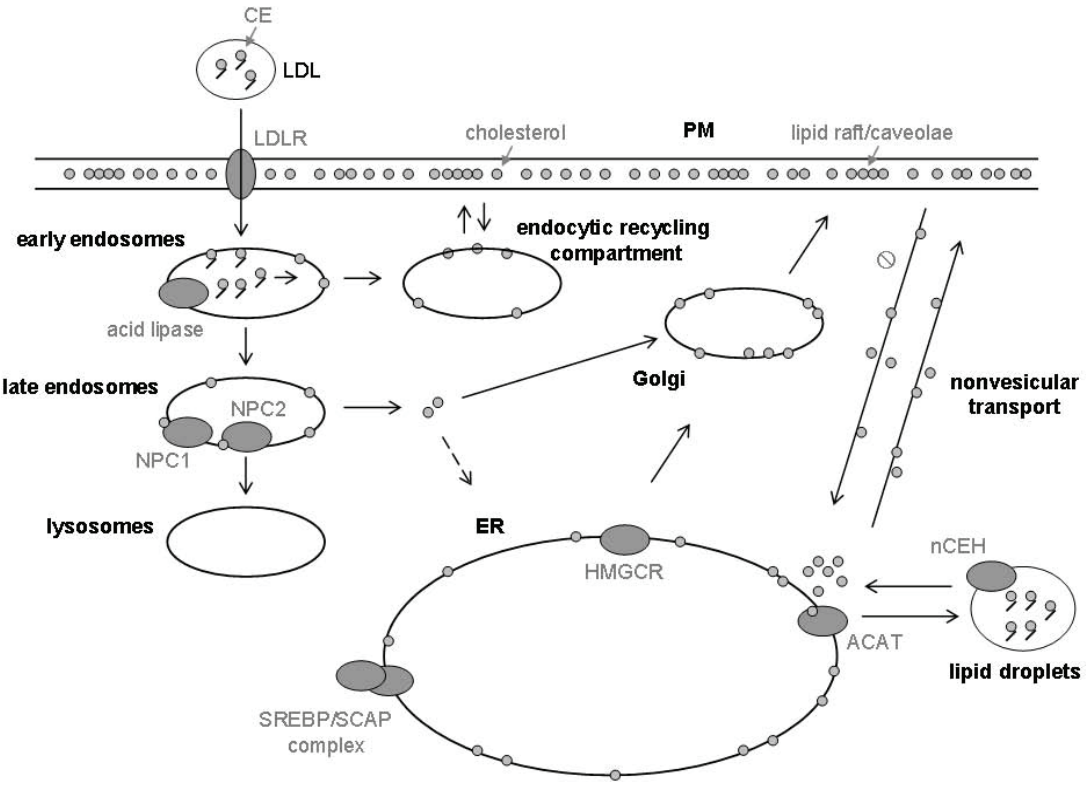

Figure 7. A model for intracellular cholesterol trafficking in mammalian cells.

2.5.4. Cholesterol efflux

Cholesterol synthesized in the ER has to move to towards the plasma membrane against a steep concentration gradient (Prinz, 2002; Soccio and Breslow et al., 2004). This movement may occur through either by a vesicular or a non-vesicular transport. Data supporting the idea of vesicular transport, involving the protein secretory pathway through the Golgi, is that ATP depletion or low temperature rapidly inhibits cholesterol transport from the ER to the plasma membrane. Nevertheless, brefeldin A treatment, which causes Golgi disassembly and fusion with the ER, blocks protein secretion but only decreases cholesterol transport to the plasma membrane slightly. Therefore, vesicular transport through the Golgi may transfer some nascent cholesterol to the plasma membrane but it is not the major pathway. As far as non-vesicular movement is concerned, there are data to support the idea

that cytosolic proteins such as sterol carrier protein-2 (SCP-2) or caveolin are involved in the process, but the precise role of either of these is inconclusive (Prinz, 2002).

Not only newly synthesized cholesterol but some precursors as well as other biosynthetic sterols, — such as zymosterol, lathosterol or desmosterol — are shown to be quickly targeted to the plasma membrane, becoming then available to extracellular acceptors (Lusa et al., 2003). In yeast, Osh proteins have been implicated in sterol transport from ER to plasma membrane, but rather than being sterol transporters, it was proposed that these proteins influence sterol trafficking by affecting the ability of the plasma membrane to sequester sterols (Sulivan et al., 2006).

When cholesterol accumulates in the plasma membrane it can not only be internalized back towards organelles but also can be effluxed from cells in a specific extracellular/intracellular scenario. Cellular cholesterol efflux is a critical event for cellular cholesterol homeostasis as cells can alleviate the excess of cholesterol by its release from the cell. Cholesterol efflux occurs by two distinct mechanisms (Yokoyama, 2005): (*a*) passive diffusion, driven by the cholesterol gradient between the cell surface and the high density lipoprotein (HDL) in contact with the cell surface, and (*b*) an apolipoprotein-mediated pathway that generates nascent HDL particles, using mainly the apoA-I (which is synthesized mostly in the liver) and phospholipids and cholesterol of peripheral cells as the substrates.

The ABC protein ABCA1, located mainly at the plasma membrane of peripheral cells, plays an essential role in efflux process. Tangier Disease, a rare disease consisting in HDL deficiency, is caused by defective mutations within the coding regions of the *Abca1* gene (Oram, 2002). How ABCA1 mediates cholesterol efflux is under study. Some findings show that ABCA1 gene expression is mainly controlled by transcription and by protein degradation. For transcriptional control, the liver X receptors (LXRs) bind to oxidized derivatives of cholesterol called oxysterols and form heterodimers with the retinoid X receptors (RXR), which use retinoic acid as their natural ligand (Repa and Mangelsdorf, 2000). The heterodimeric complex then becomes active in stimulating expression of the *Abca1* gene (Venkateswaran et al., 2000) as well as of other genes involved in cellular sterol efflux (Chawla et al., 2001). On the other hand, binding of ABCA1 with apolipoproteins stabilizes ABCA1 against degradation (Yokoyama, 2005).

As stated above, there exist a relationship between nascent cholesterol or stored cholesterol and its efflux. The removal of cellular cholesterol by ApoA-I mobilizes certain intracellular cholesterol pools and causes a decrease in ACAT activity in intact cells. Moreover, blocking ACAT activity with specific inhibitors expands the cellular cholesterol pool available for ApoA-I-mediated cholesterol efflux (Chang et al. 1997, Yamauchi et al. 2004).

Recent studies show that in macrophages, in addition to ABCA1 transporter, ABCG1 mediates cellular cholesterol efflux to mature HDL by passive diffusion and results in a net reduction in cellular cholesterol (Wang et al., 2004, Kennedy et al., 2005). It is thought that ABCG1 cooperates with ABCA1 by further adding cellular lipids to the nascent particle, which results in the maturation of HDL. In this sense, some recent data indicate that ABCA1 and ABCG1 synergize to mediate cholesterol export to ApoA-I (Gelissen et al., 2006). Targeted disruption of macrophage ABCA1 and ABCG1 leads to a complete impediment of cholesterol efflux in vitro, and an in vivo accelerated atherosclerosis (Yvan-Charvet et al., 2007).

In cells of peripheral tissues, excess cellular cholesterol needs to be removed and transported to the liver for reutilization and excretion. This process is collectively termed reverse cholesterol transport, and is briefly represented in Figure 8. The HDL acts as the major acceptor for cellular cholesterol released from the extrahepatic cells. The mature globular HDLs transport CE to the liver, the adrenals glands, and other steroidogenic tissues. At the surface of the liver cells and steroidogenic cells, the HDL is recognized by the HDL receptor, named scavenger receptor type B class I (SRB1) (Krieger, 1999; Azhar and Reaven, 2002). The SRB1 mediates internalization of CE by a selective uptake process and thus CE are hydrolyzed to free cholesterol. The compartment involved the CE hydrolysis that enter through this pathway is not known but it does not involve NPC1 (Xie et al., 2000). In the liver, the HDL-derived cholesterol serves as an important precursor for bile acid synthesis; it can also enter the bile duct, to be excreted from the body. As an example, SRB1 expression correlates with biliary cholesterol secretion, in a not completely known manner (Harder et al., 2007). In steroidogenic tissues, the HDL-derived cholesterol serves as an important precursor for steroid hormone synthesis. HDL is recognized as an antiatherogenic lipoprotein; raising

plasma HDL is a potential therapeutic goal for treating atherosclerotic cardiovascular disease (Linsel-Nitschke and Tall, 2005).

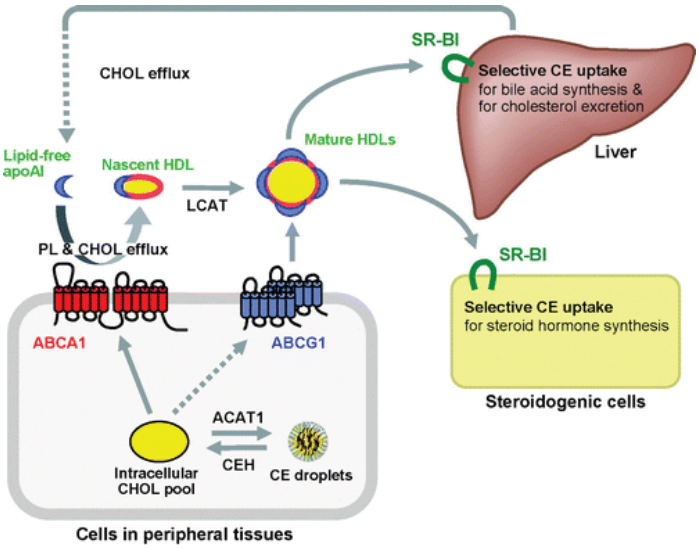

Figure 8. Reverse cholesterol transport in mammals. Cells in peripheral tissues export cholesterol/CE to apoproteins, by mainly ABCA1 and ABCG1 transporters, forming HDLs. HDLs reach steroidogenic cells and liver and are internalized via SRB1 to form steroid hormones and bile acid correspondingly. Some abbreviations used: Lecithin:cholesterol acyltransferase (LCAT), Cholesteryl ester hydrolase (CEH), Phospholipids (PL), Cholesterol (CHOL). From Chang et al., 2006.

2.5.5. Regulation of cholesterol homeostasis

It is known that both the biosynthesis and uptake of cholesterol are well regulated by feedback control. When cells accumulate excess sterols, the activity of HMGCR declines by more than 90% and the number of LDLRs also decreases. In contrast, when intracellular cholesterol is depleted the activity of this enzyme remains high and the cells express a large number of LDLRs on their surface (Goldstein et al., 2006). Moreover HMGCR protein levels are reduced by lipoprotein derived cholesterol. Indirect evidence from some reports (Brown and Goldstein, 1975; Golstein and Brown 1984) suggested that the major mechanism for the decrease of HMGCR and LDLR levels is a reduction in the mRNAs for these two proteins. Some other experiments a time later indicated the presence of a transcription factor that promotes expression of both proteins-associated genes. In 1990 it was located a 10-bp sequence that was named the sterol regulatory element (SRE), thought to be target of the unknown transcription factor (Smith et al., 1990).

In the process for understanding the genetic regulation of cholesterol homeostasis Joseph L. Goldstein´s laboratory designed an antibody against a specific protein that contains

in its sequence an SRE binding domain, which had been named as SRE binding protein (SREBP). When they applied the antibody to cultured fibroblasts incubated with or without exogenous cholesterol, in sterol-depleted cells the SREBP was located into the nucleus, whereas in sterol-exposed cells it exhibited a different location consistent with ER membranes (Wang et al., 1994). Two different SREBP proteins have been reported: SREBP-1, which has two isoforms -1a and -1c and SREBP-2. SREBP-1 has shown to be related with fatty acid synthesis, while SREBP-2 regulates genes of cholesterol biosynthesis and uptake.

SREBP-2 has two different states that differ in molecular weight and location (Yokoyama et al., 1993). The mature 60 KDa state, found in the nucleus, and a 125 KDa protein, that is located in ER. Moreover, the 60KDa isoform correspond to the N-terminal portion of the 125 KDa protein. The sequence of the unprocessed 125 KDa SREBP has shown to contain the following elements: 1) an N-terminal fragment that contains a basic-helix-loop-helix-leucine zipper domain (bHLH-Zip) 2) a 80 amino acid sequence containing two membrane-spanning helices separated by a 30 amino acid hydrophilic sequence. 3) a ~550 amino acid sequenced named regulatory domain. The N-terminal bHLH-Zip domain and the C terminal regulatory domain project into the cytosol whereas the 30-amino acid hydrophilic loop is exposed to the lumen of ER (Fig. 9).

Some studies revealed that, for the processing of the inmature isoform towards transcription factor 60 KDa peptide, two proteolytic cleavages are required (Sakai et al., 1996) and these are done by two different proteases. The first protease cuts SREBPs at a conserved leucine residue within the 30-amino acid luminal loop. This protease, called site-1 protease (S1P), separates the SREBPs into two halves but keeping the bHLH-Zip domain associated with the membrane. After cleavage by S1P, a second protease, designated site-2 protease (S2P), clips the N-terminal fragment, releasing the bHLH-Zip domain from the membrane. Interestingly, the active form these proteases are located at Golgi (Epenshade et al., 1999), which suggests that SREBP needs to travel to this organelle for its maturation. Because of that, the SREBP cleavage showed to need the involvement of a SREBP cleavage-activating protein (SCAP). Fluorescence microscopy demonstrated that this protein was localized to the ER in sterol-loaded cells, and it moves rapidly to the Golgi complex when sterols are depleted. More specifically, there exist a Scap/SREBP complex that moves from ER to Golgi in a sterol-sensitive fashion (Nohtufft et al., 1998) (Fig. 9).

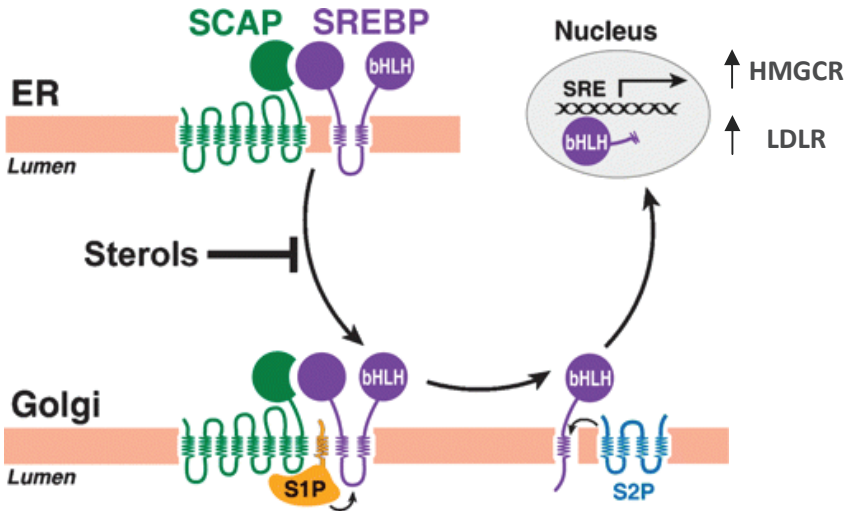

Figure 9. SREBP location pre/post protease cleavages. When sterol levels are high inside the cell, unprocessed SREBP is located in ER with its regulatory and bHLH-Zip domains exposed to cytosol and SP1 cleavage domain towards ER lumen. When sterol levels are decreased, the complex SCAP/SREBP goes to Golgi where proteases SP1 and SP2 cleave SREBP, releasing the mature transcription factor to the nucleus. Taken from Brown and Goldstein, 2009.

But, how sterol regulates the SCAP/SREBP delivery to Golgi? For the understanding of this process, a new protein came into scene – this is Insig, which stands for insulin-induced gene (Diamond et al., 1993). Insig was identified as an SREBP-induced target gene in CHO cells, so that is related with SREBP activation; there exist two Insig isoforms, Insig-1 and Insig-2, being both required for sterols to retain the Scap/SREBP complex in the ER (Adams et al., 2004). Scap binds to Insig only in the presence of sterols, either cholesterol or 25-hidroxycholesterol (Yang et al., 2002; Adams et al., 2004). It was found that Scap has different conformation when it is isolated from sterol-deprived cells when compared to sterol-treated ones. Moreover, the conformational change involves arginine-503, belonging to a hexapeptide MELADL, which resides in a loop located in Scap protein (Fig. 10.A). Interestingly, the modulation of SCAP/SREBP complex delivery differs depending on the kind of sterol, while cholesterol binds to Scap, oxysterols bind to Insig. Either of these facts however entails binding of Scap and Insig so that MELADL peptide is not accessible to proteins Cop II. The function of these proteins is to target MELADL and sequester

Scap/SREBP towards Golgi, but only in low-sterol situation (figure 10.B). Cop II/Scap/SREBP vesicles are directed to Golgi where proteases SP1 and SP2 release SREBP mature transcription factor that goes to nucleus to enhance expression on SRE-containing genes such as HMGCR or LDLR.

Regarding proteins degradation, it has been described that when sterol levels are high Scap has the ability to bind to HMGCR in ER. This binding promotes HMGCR ubiquitination and consequent degradation, so that cholesterol biosynthesis is both, transcriptionally and enzymatically impeded in high sterol context (Goldstein et al., 2006).

As summary, once the capacity of the plasma membrane and other compartments to absorb cholesterol is exceeded, cholesterol is transported back to the ER, where it is esterified by ACAT, regulates HMGCR proteolysis and inhibits the proteolytic processing of sterol SREBP-2, which is required for the expression of sterol-regulated genes (Goldstein et al., 2006; Sato, 2010). The main regulatory mechanism is the sensing of intracellular cholesterol in the ER by the protein SCAP. In the absence of cholesterol the mature SREBP-2 migrates to the nucleus and acts as a transcription factor to stimulating the transcription of many genes (Sato, 2010), among which are those encoding LDLR and HMGCR.

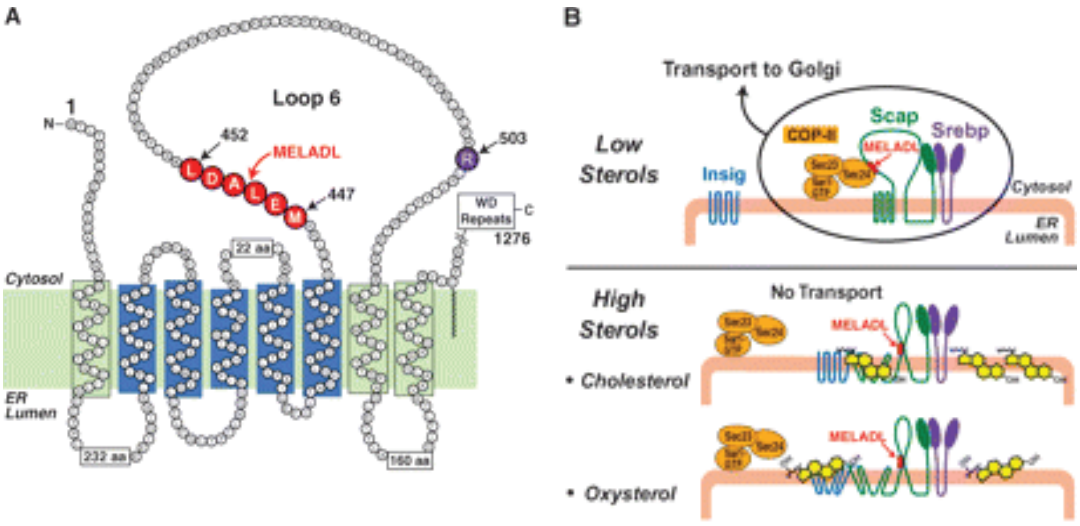

Figure 10. SCAP structure and conformational change. A. A hamster SCAP sequence and topology, showing MELADL hexapeptide. B. When cholesterol binds to SCAP or oxysterol binds to Insig, MELADL is exposed to Cop II proteins, which transport the complex to Golgi. From Brown and Goldstein, 2009.

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Objectives

3. Objectives

Lipid modulation is acquiring importance in cancer treatment and thence membrane-targeted lipid analogs are currently used in clinical trials either directly or as a complement of cancer therapy. As exposed under Introduction, there is some background that links APLs with cholesterol metabolism. Since cholesterol has shown to be determinant in cancer progression, we propose that its metabolism is a target of lipid analogs with antitumoral activity. The development of APLs and their use in clinics, evidence the need for an understanding of the mechanism by which these agents exert their action and how the sterols are related with its biologic activity.

The **main objective** of this thesis is to deeply study the actions of APLs on cholesterol metabolism and homeostasis, focusing in events like its synthesis, location, trafficking and efflux.

To achieve this aim, we have proposed the following **specific objectives**:

1) Test the antiproliferative activity of APLs on tumor human cell lines HepG2 and U-87 MG and make a comparative study of their inhibitory potencies.

2) Determine the actions of APLs on cell cycle and related processes such as autophagy; detect if treatment with lipid analogs causes any ultrastructural anomalies.

3) Perform studies of cholesterol biosynthesis and accumulation inside the cells.

4) Establish the effects of APLs on cholesterol internalization, transport and esterification.

5) Determine how APLs modulate raft micro-domains by its isolation with a free-detergent method and subsequent study of its lipid profile; analyze the cholesterol/sphingolipid ratio.

6) Study lipid efflux from cells to extracellular medium in presence of APLs and probe how lipofilic acceptors could modulate this activity.

7) Perform lipid transcriptome and proteome studies to check the effect of APLs on cholesterol anabolism, catabolism, internalization and efflux.

8) Study the modulation of PI3K/AKT signaling survival pathway by APLs through phosphoinositides levels and AKT phosphorylation; test the reversibility of the process by cholesterol replenishment.

Results

4. Results

4.1. Hexadecylphosphocholine alters nonvesicular cholesterol traffic from the plasma membrane to endoplasmic reticulum and inhibits the synthesis of sphingomyelin in HepG2 cells

In this first work we focused in the APL analogue miltefosine or HePC and the effect of this compound in the intracellular cholesterol trafficking inside the hepatoma HepG2 cell line. Cholesterol sensing mechanisms are mainly located in the ER, where excess of cellular cholesterol is converted to cholesteryl esters by the enzyme ACAT. We have centered this study in cholesterol and sphingolipid synthesis and transport.

Firstly, we labeled cells with radioactive acetate, which is incorporated to cholesterol and biosynthesic intermediates through the cholesterogenic pathway. We detected that HePC promoted an increase in some intermediates of cholesterol biosynthesis as desmosterol and 7-dehydrocholesterol, suggesting a cholesterol biosynthetic activation. On the contrary, we found that sphingomyelin and ceramide synthesis were significantly inhibited in HepG2 cells after exposure to HePC.

Radiolabeling and tracking of cellular cholesterol helped us to analyze the effect of HePC on cholesterol transport in three different directions: 1) LDL-cholesterol uptake, internalization and transport back to plasma membrane, 2) Transport of newly synthetized cholesterol from ER to plasma membrane, 3) direct cholesterol transport from plasma membrane to ER. HePC produced a noteworthy inhibition of the last kind of transport (from plasma membrane to ER), but no significant effect for either of the two others. Moreover, experiments performed in ATP depleted cells show that HePC inhibits cholesterol transport from plasma membrane to ER with similar accuracy than with available ATP. This last result suggests that this agent alters only the non vesicular energy-independent cholesterol traffic without altering the vesicular transport. In addition, hydrolysis of plasma membrane sphingomyelin by exogenously added sphingomyelinase resulted in enhanced plasma-membrane cholesterol esterification, but this treatment did not prevent the inhibition in cholesteryl ester formation caused by HePC.

Summarizing, HePC affects significantly the cholesterol and sphingomyelin homeostasis in HepG2 cells. This alteration could be related with the antiproliferative activity reported previously by our research group and others. Moreover, since sphingomyelin and cholesterol are major lipid constituents of membrane raft microdomains, these results suggest that HePC could disturb membrane raft integrity and thence its functionality and so, further studies on this direction need to be done.

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Hexadecylphosphocholine alters nonvesicular cholesterol traffic from the plasma membrane to the endoplasmic reticulum and inhibits the synthesis of sphingomyelin in HepG2 cells

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ABSTRACT

The synthetic lipid analogue, hexadecylphosphocholine is an antitumoral and antileishmanial agent that acts on cell membranes and can induce apoptosis. We have previously investigated the effect of hexadecylphosphocholine on the biosynthesis and intracellular transport of cholesterol in the human hepatoma HepG2 cell line. Here we show that the traffic of endocytosed-cholesterol from LDL to the plasma membrane and the transport of newly synthesized cholesterol from the endoplasmic reticulum to the plasma membrane were unaffected by alkylphosphocholine exposure. On the contrary, cholesterol traffic from the plasma membrane to the endoplasmic reticulum was drastically interrupted after 1 h of cell exposition to HePC and, consequently, the intracellular esterification of cholesterol was substantially decreased. Our results also demonstrate that this alkylphosphocholine exclusively affected the nonvesicular, energy-independent cholesterol traffic, without altering the vesicular transport. In addition, hydrolysis of plasma membrane sphingomyelin by exogenously added sphingomyelinase resulted in enhanced plasma-membrane cholesterol esterification, but sphingomyelinase treatment did not prevent the inhibition in cholesteryl ester formation caused by hexadecylphosphocholine. We also found that sphingomyelin synthesis was significantly inhibited in HepG2 cells after exposure to hexadecylphosphocholine. Since sphingomyelin and cholesterol are major lipid constituents of membrane raft microdomains, these results suggest that hexadecylphosphocholine could disturb membrane raft integrity and thence its functionality.

1. Introduction

Alkylphosphocholines (APC) are a new class of antitumour agents that have been shown to induce apoptosis in several tumour cells. Hexadecylphosphocholine (HePC) is a lipid analogue belonging to the APC group which exerts antiproliferative activity against a broad spectrum of established tumour cell lines (Wieder et al., 1998). This lipid analogue also shows potent leishmanicidal activity as a consequence of its interference in the parasite's metabolic pathways and the induction of apoptosis (Soto and Soto, 2006; Rakotomanga et al., 2007).

HePC has amphiphilic properties and thus interacts with the cell membrane and rapidly reaches other subcellular membranes (Berkovic et al., 2003), thus being able to affect cell metabolism at different levels. We reported in previous studies that HePC interferes with phosphatidylcholine (PC) synthesis in HepG2 cells via both CDP-choline (Jiménez-López et al., 2002) and phosphatidylethanolamine methylation (Jiménez-López et al., 2004). Recently, we have established that HePC also alters intracellular cholesterol metabolism leading to an increased uptake, synthesis and accumulation of cholesterol in the cell (Jiménez-López et al., 2006; Carrasco et al., 2008).

Sterols are critical for eukaryotic cell membrane function due probably to their key role in lipid rafts. The list of raft-dependent functions in cells is continuously expanding. Cholesterol and lipid rafts not only participate in distributing proteins to the cell surface and to other organelles, but they also play a significant role in many signaling cascades (Simons and Toomre, 2000) and in the activation of immune responses (Langlet et al., 2000). However, cholesterol is not solely beneficial to the cell. Excess cholesterol is toxic and therefore cells have to employ a number of safety devices

Abbreviations: APC, alkylphosphocholine; CE, cholesteryl esters; CL, cholesteryl linoleate; ER, endoplasmic reticulum; FBS, fetal bovine serum; HePC, hexadecylphosphocholine; HMG, 3-hydroxy-3-methylglutaryl; 2OHpβCD, 2-hydroxypropyl-β-cyclodextrin; MβCD, methyl-β-cyclodextrin; LDL, low density lipoprotein; MEM, minimal essential medium; PC, phosphatidylcholine; SCAP, SREBP cleavage-activating protein; SM, sphingomyelin; SMase, sphingomyelinase; SREBP, sterol regulatory element-binding protein.

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to limit the concentration of free cholesterol. Breakdown of cholesterol homeostasis causes disease states, being the most common atherosclerosis or Alzheimer's disease.

Three organelles are involved in cholesterol trafficking: (1) endoplasmic reticulum (ER), the major site of synthesis, regulation and esterification of cholesterol, (2) plasma membrane, a prominent storage site for free cholesterol, and (3) lysosomes, where lipoprotein-derived cholesterol is liberated. Cholesterol made in the ER, as well as that released in the lysosome by lipoprotein catabolism, moves to the plasma membrane (reviewed by Soccio and Breslow, 2004). Once the capacity of the plasma membrane to absorb cholesterol is exceeded, cholesterol is transported to the ER, where it is esterified, regulates 3-hydroxy-3-methylglutaryl (HMG)-CoA reductase proteolysis, and inhibits proteolytic processing of sterol regulatory element-binding protein (SREBP) required for expression of sterol-regulated genes (Goldstein et al., 2006). This distribution of cholesterol between sites of regulation, synthesis, and deposition provides for efficient control of cellular cholesterol levels. With the exception of the SREBP cleavage-activating protein (SCAP) system, the complex control mechanisms for regulating cholesterol levels in different cellular compartments remain largely unknown.

In the present work, we extend our studies to further analyze the effects of HePC on cholesterol metabolism and intracellular traffic. We report here that HePC interferes with the transport of cholesterol from the plasma membrane to the ER which is observable even in a 60 min period of HePC treatment. On the contrary, this APC has no effect on the arrival of low density lipoprotein (LDL)-cholesterol to the plasma membrane or the movement of newly synthesized cholesterol from the ER to the plasma membrane.

2. Materials and methods

2.1. Chemicals and reagents

Radiolabeled compounds were supplied by American Radiolabeled Chemicals (St Louis, MO, USA). Fetal bovine serum (FBS) was from The Cell Culture Company (Pasching, Austria). Minimal essential medium (MEM), bacterial sphingomyelinase (SMase) and TLC plates were from Sigma–Aldrich (Madrid, Spain). HePC was supplied by Cayman Chemical (Ann Arbor, MI, USA). All other reagents were of analytical grade.

2.2. Cell culture

The human hepatoma HepG2 cell line was from The European Collection of Animal Cell Cultures (Salisbury, UK). Cells were cultured in MEM containing 10% heat-inactivated FBS, supplemented with 2 mM L-glutamine, 1% nonessential amino acids, 100 U ml^{-1} penicillin and 100 $\mu g\,ml^{-1}$ streptomycin. Cells were grown in a humidified atmosphere with 5% CO_2 at 37 °C and were subcultured at a 1:10 ratio once a week. Cells were plated on tissue-culture dishes (Nunc™) at a density of 5×10^4 cells cm^{-2} and maintained in culture medium for 3 days before being used in experimental radiolabeling assays at approximately 70% confluence.

2.3. Determination of sterol biosynthetic intermediates

HepG2 cells growing in log phase were cultured in MEM/10% FBS and treated for 6 and 24 h with 50 μM HePC. To label newly synthesized cholesterol, MEM containing [1-^{14}C]acetate (87 μM, 58 Ci mol^{-1}) was added to the cells and the incubation was continued for 1 h. Then, cells were washed twice with ice-cold PBS to remove unincorporated radiolabel and cholesterol intermediates were determined by the method described by Yamauchi et al. (2007). Briefly, cells were dissolved in 2 ml of 2 M NaOH and transferred to glass tubes. An equal volume of 95% ethanol was added and the solution was heated at 80 °C overnight. After saponification, each sample was evaporated under N_2 until the volume was reduced to 1 ml. The volume was then brought back to 2 ml with water, and the nonsaponifiable lipid fraction was extracted four times with 4 ml of nanograde petroleum ether. The petroleum ether fraction was pooled, concentrated under N_2 to 2 ml, and washed twice with 1 ml of 3% $NaHCO_3$ and once with 1 ml of water. The solvent was evaporated under N_2 and the lipid extract was used to separate cholesterol and its intermediates by TLC. To isolate C27 sterols (cholesterol, lathosterol, 7-dehydrocholesterol and desmosterol) we carried out a run in chloroform. After chromatography, the plates were briefly stained with iodine vapour to identify the C27 sterol band that was scraped and extracted with chloroform/methanol (2:1, v/v) twice. The extracts were transferred to new glass tubes, washed once with water, and dried under N_2. The C27 sterols were separated by TLC using plates impregnated with 10% silver nitrate. The samples were chromatographed using chloroform as solvent. Plates were run with authentic sterol intermediates spotted in parallel lanes as standards. After TLC, the standard bands were identified by spraying the plates with 0.2% fluorescein in ethanol. Radiometric measurements of scraped lipids were made by liquid scintillation using a Beckman 6000-TA counter (Madrid, Spain).

The Rf value of the sterols analyzed is: for the chromatography used for the separation of C29/C30 and C27 sterols; C27 sterols ~0.15. For the system used to separate C27 intermediates: 7-dehydrocholesterol ~0.03, desmosterol ~0.06, cholesterol ~0.09, lathosterol ~0.12.

2.4. Metabolic labeling assays

The synthesis of sphingomyelin (SM) and ceramides was determined by measuring the incorporation of radioactive exogenous palmitate into cellular SM and ceramides. Log-phase HepG2 cells were incubated in medium containing 10% FBS either with 50 μM HePC for 6 and 24 h or without (control). [9,10-^{3}H]Palmitate (100 μM, 34 Ci mol^{-1}) was added during the last 6 h of incubation. After labeling, the medium was collected and the cells were washed twice with ice-cold PBS before being harvested by scraping into PBS. The lipids were extracted from the cells following the procedure of Bligh and Dyer (1959). SM and PC were separated by TLC using a mixture of chloroform/methanol/acetic acid/water (60/30/8/5, v/v/v/v) as solvent. Ceramides were separated in chloroform/methanol (9/1, v/v). The spots were rendered visible by exposure to iodine vapour.

To assay the effect of HePC and SMase treatment on PC and SM levels, HepG2 cells were labeled to equilibrium during 36 h with [methyl-^{14}C]choline (18 μM, 56 Ci mol^{-1}). After labeling period, cells were washed with PBS and then fed with medium with or without 50 μM HePC and/or 50 mU ml^{-1} SMase and incubated for 60 min at 37 °C. SM and PC were separated as described above.

To analyze the incorporation of [9,10 (n)-^{3}H]oleate into cholesteryl oleate, HepG2 cells were treated in MEM/10% FBS with or without 50 μM HePC and/or 50 mU ml^{-1} SMase and incubated for 60 min at 37 °C in the presence of [9,10 (n)-^{3}H]oleate (100 μM, 34 Ci mol^{-1}). Cholesteryl [^{3}H]oleate was isolated from cells by TLC using hexane/diethyl ether/acetic acid (80/20/2, v/v/v) as solvent. Radiometric measurements of scraped lipid spots were made by liquid scintillation.

2.5. Trafficking of newly synthesized cholesterol to the plasma membrane

Trafficking of newly synthesized cholesterol from the ER to the plasma membrane was estimated by availability of newly syn-

thesized cholesterol for efflux at the plasma membrane, using cyclodextrin as acceptor. Briefly, HepG2 cells were treated with 50 μM HePC for 6 or 24 h. After treatment, cells were incubated for 60 min with [1-^{14}C]acetate (87 μM, 58 Ci mol^{-1}) to label the newly synthesized cholesterol. Then, cells were washed three times with ice-cold PBS to remove unincorporated radiolabel. After washing, cells were incubated for 10 min at 37 °C with 20 mM 2-hydroxypropyl-β-cyclodextrin (2OHpβCD) or for 30 min at 37 °C with 10 mM methyl-β-cyclodextrin (MβCD). Cholesterol from the cells and medium was isolated by TLC using hexane/diethyl ether/acetic acid (80/20/2, v/v/v) as solvent. Radiometric measurements of scraped lipid spots were made by liquid scintillation. The percent cholesterol efflux was calculated as [^{14}C]cholesterol counts in the medium divided by the sum of [^{14}C]cholesterol counts in the cells plus in the medium.

2.6. Assays to monitor the destiny of [^{3}H]cholesteryl linoleate in LDL

[1α,2α(n)-^{3}H]cholesteryl linoleate ([^{3}H]CL) was incorporated into LDL using a modification of the procedure described by Sugii et al. (2006). Cells were plated in MEM/10% FBS in 12-well dishes and then were cultured for 24 h in FBS-free MEM to deplete stored cholesterol within the cell. Prior to labeling, cells were pre-chilled on ice, then labeled with 20 μg/ml [^{3}H]CL-LDL in MEM for 5 h at 18 °C. After incubation, cells were washed once with ice-cold PBS containing 1% BSA, and washed three more times with ice-cold PBS. Cells were then incubated with medium with or without 50 μM HePC for 60 min at 37 °C in the presence of 10 mM MβCD. Afterwards, lipids from cells and medium were extracted and analyzed as described above. The percent of cholesterol efflux was calculated as [^{3}H]cholesterol counts in the medium divided by the sum of [^{3}H]CL counts in the cells and [^{3}H]cholesterol counts in the cells plus in the medium.

2.7. Trafficking of cholesterol from the plasma membrane to the endoplasmic reticulum

An appropriate way to measure cholesterol transport from the plasma membrane to the ER is to determine the degree of esterification of radiolabeled cholesterol previously incorporated into the plasma membrane (Lange and Steck, 1997). Thus, HepG2 cells were labeled with 2 μCi of [7(n)^3-^{3}H]cholesterol (dissolved in 2-propanol) for 1 h at 37 °C to label the plasma membrane. To remove any unincorporated radioactivity, the cells were washed twice with PBS containing 0.5% BSA prewarmed to 37 °C. The cells were then incubated at 37 °C in MEM for 60 min in the presence or absence of 50 μM HePC and/or 50 mU ml^{-1} SMase. Then, the medium was removed and the lipids were extracted and analyzed as described above.

To determine if cholesterol movement from the plasma membrane to the ER depends on energy, we conducted similar experiments in which ATP depletion was performed preincubating cells during 1 h in a glucose-free Ringer's solution containing 10 mM 2-deoxyglucose and 15 μM sodium azide.

2.8. Statistics

The results were expressed as means ± SEM. One-way ANOVA with subsequent post hoc comparisons by Scheffe's test was carried out (SPSS 13.0). *P* values <0.05 were considered to be statistically significant.

2.9. Other analyses

Cell protein content was determined in the cell homogenates by Bradford's method (1976) using BSA as standard.

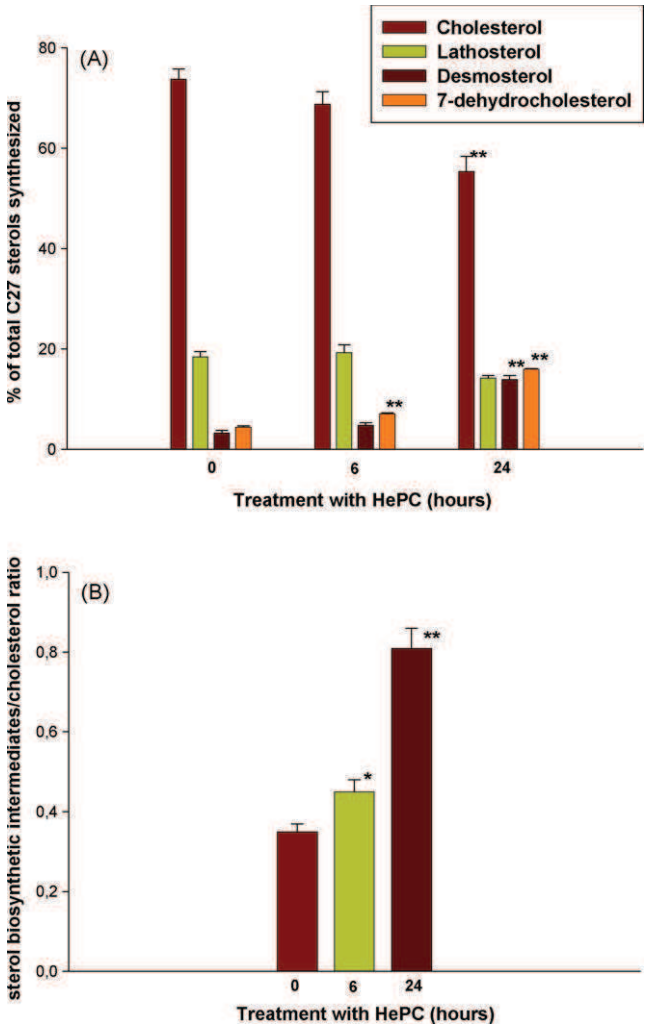

Fig. 1. Effect of hexadecylphosphocholine on intermediates of cholesterol biosynthesis in HepG2 cells. Log-phase HepG2 cells were incubated with MEM containing 10% FBS either with 50 μM HePC for 6 and 24 h or without any additions (control). [1-^{14}C]Acetate (87 μM, 58 Ci mol^{-1}) was added during the last hour of incubation. The incorporation of acetate into cholesterol and sterol intermediates was determined as described in Section 2. The results are expressed in terms of the percentage of total C27 sterols synthesized (A) or as sterol biosynthetic intermediates/cholesterol ratio (B), and represent the mean ± SEM of two independent experiments conducted in triplicate. *$P < 0.05$; **$P < 0.01$ compared with the values found in the absence of HePC.

3. Results

3.1. Effect of hexadecylphosphocholine on intermediates of cholesterol biosynthesis

It has been shown that when traffic of cholesterol from the plasma membrane to the ER is disrupted, the circuit for synthesis of the sterol is affected, so intermediates of cholesterol biosynthesis are accumulated (Metherall et al., 1996; Field et al., 2007). Thence, to address if the alterations produced by HePC in cholesterol traffic and synthesis cause the accumulation of cholesterol intermediates, we exposed HepG2 cells to 50 μM HePC for 6 and 24 h and measured the amount of [1-^{14}C]acetate incorporated into the final product and into several intermediates of its biosynthetic pathway. Our results reveal that the exposure of HepG2 cells to this APC drastically alters the pattern of radioactivity distribution. So, as it can be observed in Fig. 1A, while the percentage of radioactivity in cholesterol is clearly decreased in a time-dependent manner, the contribution of desmosterol and 7-dehydrocholesterol to the total

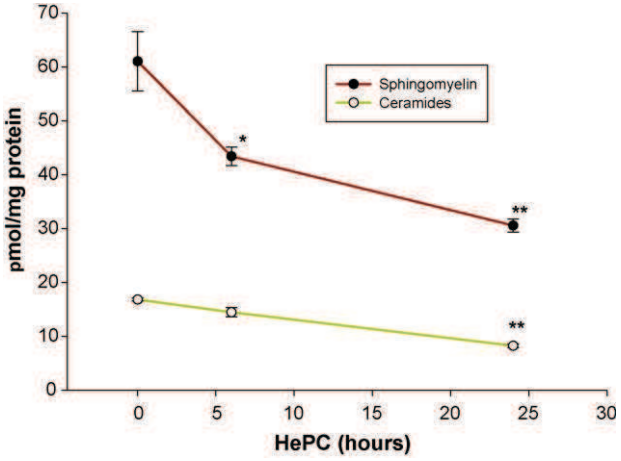

Fig. 2. Effect of hexadecylphosphocholine on sphingomyelin and ceramide synthesis from [9,10-^{3}H]palmitate in HepG2 cells. Log-phase HepG2 cells were incubated with MEM containing 10% FBS either with 50 μM HePC for 6 and 24 h or without any additions (control). [9,10-^{3}H]Palmitate (100 μM, 34 Ci mol^{-1}) was added during the last 6 h of incubation. The incorporation of palmitate into SM and ceramides was determined as described in Section 2. The results are expressed as pmol of palmitate incorporated per mg of cell protein, and represent the mean ± SEM of two independent experiments conducted in triplicate. $^*P < 0.05$; $^{**}P < 0.01$ compared with the values found in the absence of HePC.

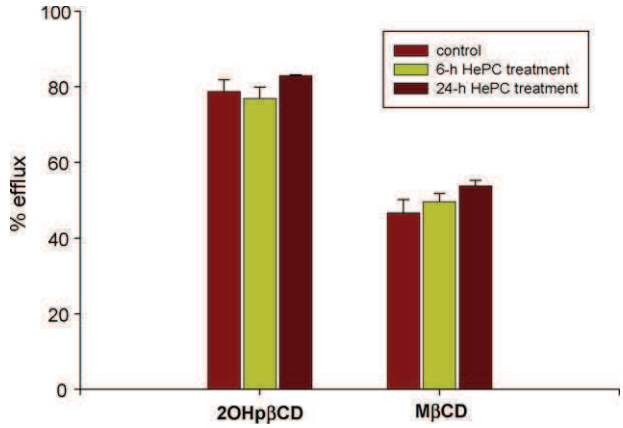

Fig. 3. Effect of hexadecylphosphocholine on trafficking of newly synthesized cholesterol from the endoplasmic reticulum to the plasma membrane. Log-phase HepG2 cells were incubated with MEM containing 10% FBS either with 50 μM HePC for 6 and 24 h or without any additions (control). [1-^{14}C]Acetate (87 μM, 58 Ci mol^{-1}) was added for 1 h to label the newly synthesized cholesterol. Trafficking of newly synthesized cholesterol from the ER to the plasma membrane was determined by using cyclodextrins as cholesterol acceptors as described in Section 2. The fraction of cholesterol moving to the plasma membrane is expressed in terms of the percentage of total label in medium versus total label in cells plus medium, and is the mean ± SEM from two independent experiments conducted in triplicate.

C27 sterol synthesis increases from an 8% in control cells up to a 30% in cells exposed for 24 h to the APC. It is interesting to note that as a consequence of these changes, the ratio of intermediates to the final product of this pathway is clearly increased in HePC-treated cells when compared to control cells (Fig. 1B).

3.2. Effect of hexadecylphosphocholine on the biosynthesis of sphingomyelin and ceramides in HepG2 cells

De novo synthesis of ceramides begins with the condensation of palmitate and serine, a reaction catalyzed by serine palmitoyltransferase. Ceramides can be further metabolized to other sphingolipids, such as SM through SM synthase. To analyze the effects of HePC on the biosynthesis of SM and ceramides, cells were treated with HePC for 6 and 24 h and labeled with [9,10-^{3}H]palmitate as exogenous precursor, as described in Section 2. As we can observe in Fig. 2, the radioactivity associated to SM is higher than that found in ceramides, indicating a high activity SM synthase. Moreover, HePC treatment produces a marked time-dependent inhibition of SM and ceramide synthesis.

3.3. Trafficking of newly synthesized cholesterol to the plasma membrane

To assay the effect of HePC on the trafficking of cholesterol from the ER to the plasma membrane, cells were incubated for 6 or 24 h with or without 50 μM HePC. [1-^{14}C] Acetate was then added and the arrival of newly synthesized cholesterol at the plasma membrane was estimated analyzing the efflux of [^{14}C]cholesterol to cyclodextrins. The high efficacy of two types of β-cyclodextrins, 2OHpβCD and MβCD for stimulating the efflux of cholesterol in HepG2 cells is shown in Fig. 3, nearly 80% and 45% of newly synthesized cholesterol was released after 10 or 30 min of incubation with 2OHpβCD and MβCD, respectively. In any case, HePC does not alter the efflux of cholesterol after 6 or 24 h exposure.

3.4. Trafficking of lysosomal cholesterol to the plasma membrane

We labeled the cells with [^{3}H]CL-LDL at 18 °C. At this temperature, LDL is internalized and accumulated in pre-lysosomal compartments without significant hydrolysis of CL (Chen et al., 1998). Upon warming to 37 °C, CL in LDL is hydrolyzed to become free cholesterol that is transported mainly to plasma membrane. By using MβCD we determined the cholesterol arriving to plasma membrane and monitored the effect of HePC on this process. As we can observe in Fig. 4, the time-course of cholesterol efflux from HepG2 cells shows that a 70% of the labeled cholesterol is readily removed in the first 2–3 min, meaning that cholesterol from LDL moves rapidly from pre-lysosomal compartments to the plasma membrane. The results also indicate that there are no differences in cholesterol arrival to the plasma membrane in HePC-treated cells with respect to control cells. Therefore, HePC does not cause cholesterol retention in endosomes/lysosomes and thus it can be delivered to the plasma membrane.

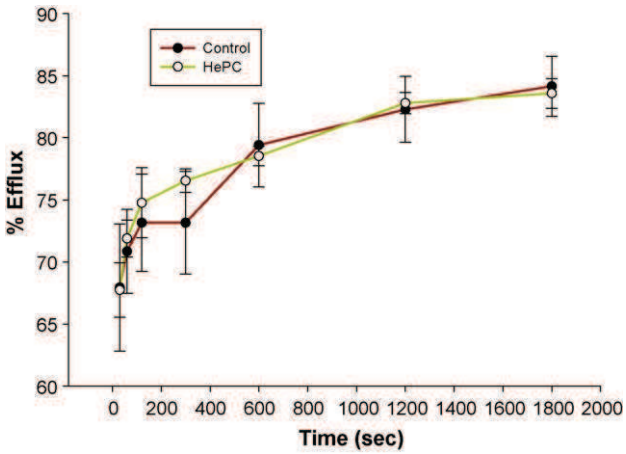

Fig. 4. Effect of hexadecylphosphocholine on transfer of lysosomal cholesterol to the plasma membrane. [1α,2α(n)-^{3}H]CL in LDL was added to HepG2 cells for 1 h in MEM either with 50 μM HePC or without any additions (control). The transport of cholesterol from pre-lysosomal compartments to the plasma membrane was determined as described in Section 2. The percent cholesterol efflux was calculated as [^{3}H]cholesterol counts in the medium divided by the sum of [^{3}H]CL counts in the cells and [^{3}H]cholesterol counts in the cells plus in the medium. The results are the mean ± SEM from two independent experiments conducted in triplicate.

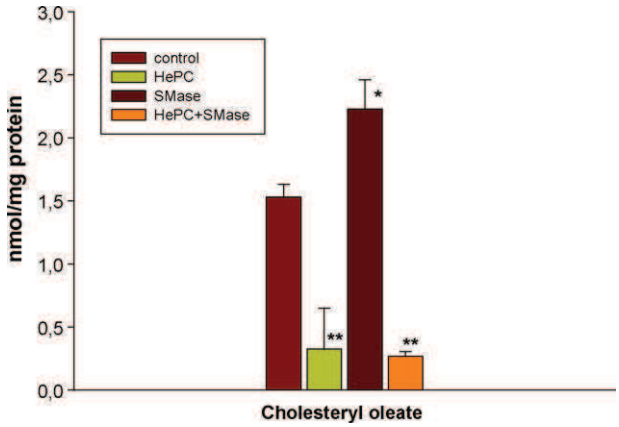

Fig. 5. Effects of hexadecylphosphocholine and sphingomyelinase on the esterification of cholesterol. Log-phase HepG2 cells were incubated with [9,10(n)-^{3}H]oleate (100 μM, 34 Ci mol^{-1}) for 1 h in serum-free MEM either with 50 μM HePC, 50 mU ml^{-1} SMase, both of them or without any additions (control). The incorporation of oleate into CE was determined as described in Section 2. The results are expressed as nmol of oleate incorporated per mg of cell protein and represent the mean ± SEM of two independent experiments conducted in triplicate. $^*P < 0.05$; $^{**}P < 0.001$ compared with control values.

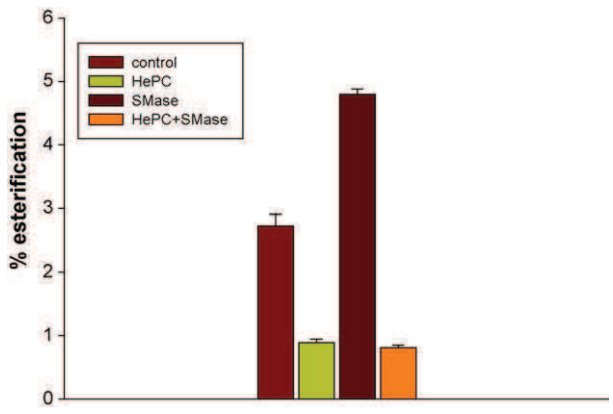

Fig. 6. Effects of hexadecylphosphocholine and sphingomyelinase on trafficking of cholesterol from the plasma membrane to the endoplasmic reticulum. Plasma-membrane cholesterol of HepG2 cells was labeled with 2 μCi [7(n)^3-^{3}H]cholesterol and cells were treated for 1 h with 50 μM HePC, 50 mU ml^{-1} SMase, both of them or without any additions (control). The fraction of plasma-membrane cholesterol that was esterified is expressed in terms of the percentage of esterification of the total labeled cholesterol and is expressed as the mean ± SEM from two independent experiments conducted in triplicate.

3.5. *Effects of hexadecylphosphocholine and sphingomyelinase on the synthesis of cholesteryl ester from [9,10 (n)-^{3}H]oleate*

Results shown in Fig. 5 demonstrate that exposure of HepG2 cells to the APC for 60 min provokes a drastic reduction in the synthesis of [^{3}H]cholesteryl oleate, since the formation of cholesteryl ester (CE) was only a 20% of that found in control cells. As previously reported in other cell lines (Zha et al., 1998), in HepG2 cells SMase treatment significantly enhances the esterification of cholesterol by a 50% with respect to control cells (Fig. 5). It is interesting to note that in cells exposed to HePC plus SMase, [^{3}H]cholesteryl oleate production was similar to that observed when cells were exposed only to HePC.

3.6. *Effects of hexadecylphosphocholine and sphingomyelinase on the utilization of plasma-membrane cholesterol for cholesteryl ester synthesis and on the levels of choline-containing phospholipids*

We used the esterification of plasma-membrane cholesterol as a marker of cholesterol movement from the cell surface to the ER, the site of acyl-CoA:cholesterol acyltransferase activity. In Fig. 6, it can be observed that the esterification of plasma-membrane cholesterol was severely impeded in HePC-treated cells, confirming that the inhibition caused by HePC in the esterification of the intracellular cholesterol pool is produced by a disruption of its movement from the plasma membrane. As expected, in cells incubated with bacterial SMase we observed a significant increase in the esterification of plasma-membrane cholesterol. It is remarkable that in cells exposed to HePC plus SMase we found a drastic reduction in cell capability to esterify cholesterol coming from plasma membrane, demonstrating that the enhanced mobilization of cholesterol produced by SMase treatment is not able to prevent the impaired arrival of labeled cholesterol to the ER induced by HePC.

These results led us to speculate if HePC could inactivate the SMase. Thence, to confirm if SMase actively hydrolyzes SM also in the presence of HePC, we carried out similar experiments labeling HepG2 cells to equilibrium for 36 h with [methyl-^{14}C]choline. After labeling the PC and SM intracellular pools, we quantified the radioactivity of both phospholipids after 60 min of HePC, SMase and HePC plus SMase treatments.

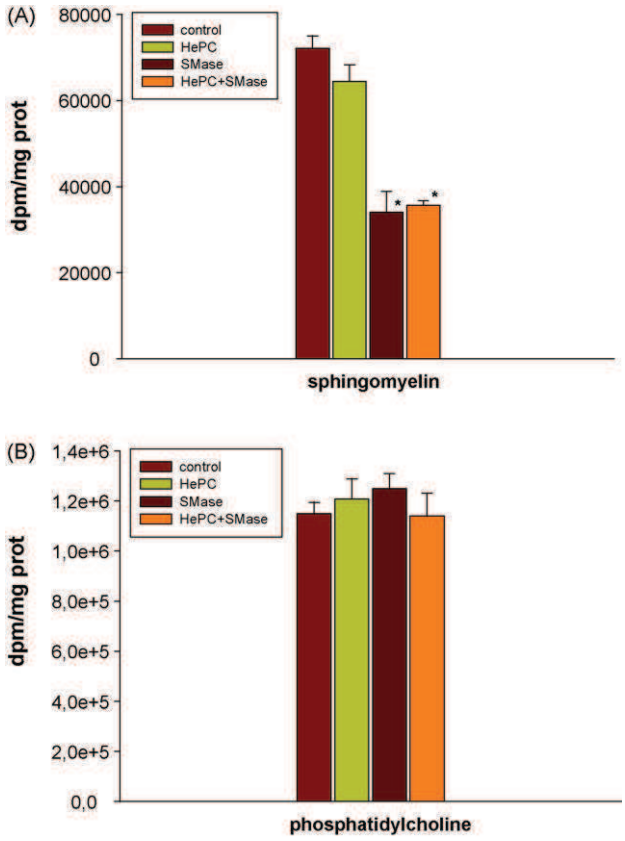

Fig. 7. Effects of hexadecylphosphocholine and sphingomyelinase on sphingomyelin and phosphatidylcholine levels. Log-phase HepG2 cells incubated with MEM containing 10% FBS were labeled to equilibrium for 36 h with [methyl-^{14}C]choline (18 μM, 56 Ci mol^{-1}). Then, cells were fed with medium either with 50 μM HePC, 50 mU ml^{-1} SMase, both of them or without any additions (control) for 1 h at 37 °C. The incorporation of choline into SM (A) and PC (B) was determined as described in Section 2. The results are expressed as dpm of choline incorporated per mg of cell protein and represent the mean ± SEM of two independent experiments conducted in triplicate. $^*P < 0.001$ compared with the values found in the absence of HePC.

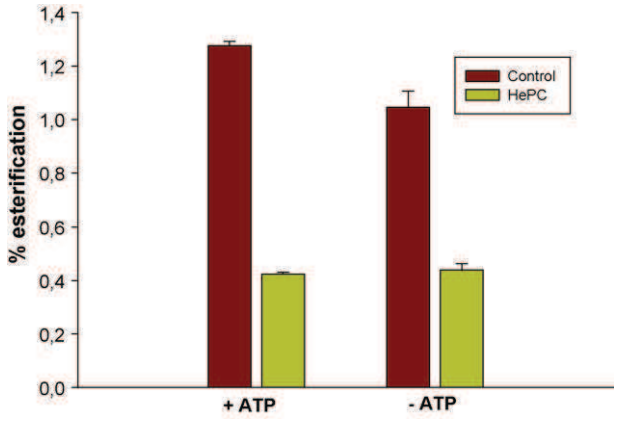

Fig. 8. Effect of hexadecylphosphocholine on cholesterol trafficking from the plasma membrane to the endoplasmic reticulum in energy-depleted cells. HepG2 cells were incubated with 2-deoxyglucose and sodium azide in order to deplete the intracellular stores of ATP as described in Section 2. Plasma-membrane cholesterol was labeled with 2 μCi [7(n)^3-^{3}H]cholesterol for 1 h either with 50 μM HePC or without any additions (control). The fraction of plasma-membrane cholesterol that was esterified is expressed in terms of the percentage of esterification of the total labeled cholesterol and represents the mean ± SEM from two independent experiments conducted in triplicate.

Results in Fig. 7A show that SMase both in the presence or absence of HePC produces a 50% decrease in the labeling of SM when compared to control cells, demonstrating that SMase efficiently catalyzes the hydrolysis of SM in HepG2 cells. In addition, in the same figure it can be seen that HePC exposure does not produce any significant effect on endogenous SMase since the label in SM was similar in control or HePC-treated cells. Furthermore, HePC and SMase treatments do not alter the label of the PC suggesting that phospholipase activities are not affected by this APC or SMase exposure (Fig. 7B).

3.7. *Effect of hexadecylphosphocholine on cholesterol transport from the plasma membrane to the endoplasmic reticulum in energy-depleted cells*

To estimate the effect of HePC on the vesicular and nonvesicular cholesterol transport from the plasma membrane to the ER, HepG2 cells were incubated with 2-deoxyglucose and sodium azide in order to deplete intracellular stores of ATP, i.e., energy-depleted cells, and then we analyzed the esterification of plasma membrane radiolabeled cholesterol.

Results showed in Fig. 8 indicate that the depletion of energy produces a decrease of 18% in the cholesterol esterification probably due to a blockage of the ATP-dependent vesicular transport. Thus, these results provide evidence that in HepG2 cells the majority of cholesterol transport from the plasma membrane to the ER is nonvesicular, energy-independent. HePC treatment induces a similar drastic decrease of cholesterol esterification in both experimental conditions, with or without ATP, indicating that this agent alters only the nonvesicular intracellular cholesterol transport.

4. Discussion

In previous works we demonstrated that concentrations of HePC less than 100 μM for 6 h have not cytotoxic effect on HepG2 cells. Moreover, non-toxic, micromolar concentrations of HePC exerted an antiproliferative effect on this hepatoma cell line. Thus, at concentrations of 50 and 75 μM of HePC we observed a decrease in the number of viable cells that was not associated by a significant increase in nonviable cells. The antiproliferative effect of HePC was accompanied by an inhibition of PC biosynthesis (Jiménez-López et al., 2002). We have previously reported that long-term HePC treatment of cells causes a marked enhancement in cholesterol synthesis (Jiménez-López et al., 2006) that has been related to an impairment in the arrival of cholesterol to the ER (Carrasco et al., 2008). Since the levels of cholesterol in the ER act as a regulatory signal that controls the cholesterogenic activity, the depletion of cholesterol leads to a deregulation in cholesterol synthesis.

Cholesterol is an essential constituent in the membrane of the mammalian cells. The cells obtain cholesterol by taking it up from their environment, mostly in the form of LDL or by de novo synthesis (Soccio and Breslow, 2004). Cholesterol biosynthesis involves successive enzymatic reactions converting the simple, 2-carbon precursor acetyl-CoA into the 27-carbon, apolar molecule cholesterol. Previous studies showed that, in addition to synthesizing cholesterol, mammalian cells also synthesize substantial amounts of precursor sterols (Echevarria et al., 1990; Lange et al., 1991). Similar to cholesterol, the precursor sterols leave the ER and rapidly reach the plasma membrane (Heino et al., 2000; Field et al., 2007) and then move back to the ER to be enzymatically processed to cholesterol, being this movement essential to complete cholesterol biosynthesis. In fact, it has been shown that agents disrupting the traffic of cholesterol from the plasma membrane to the ER lead to an accumulation of intermediates of cholesterol because the circuit for synthesis of this sterol is incomplete (Metherall et al., 1996; Field et al., 2007). In our study, cells incubated with HePC for 6 and 24 h significantly accumulated sterol intermediates producing an increase in the ratio intermediates/cholesterol. These results suggest that the APC not only interferes with the intracellular cholesterol traffic but also with the transport of these intermediates back to the ER for completion of cholesterol synthesis. Probably, both of these effects may contribute to the over-regulation of the HMG-CoA reductase and LDL receptor previously demonstrated by us (Carrasco et al., 2008).

Movement of plasma-membrane cholesterol to the ER is different from the pathway of cholesterol trafficking from the ER to the plasma membrane (reviewed by Soccio and Breslow, 2004). Cyclodextrins are widely used to analyze the arrival of newly synthesized cholesterol at the plasma membrane. These compounds are water-soluble cyclic oligosaccharides that have the capacity to sequester cholesterol in their hydrophobic cavity, thereby removing cholesterol from the cell surface rapidly and efficiently. When cyclodextrins were added to the culture medium, the majority of cholesterol located in the plasma membrane is largely susceptible to extraction. Thence, to assay transport of newly synthesized cholesterol from the ER to the plasma membrane we used 2OHpβCD and MβCD that when added to the cells for a short time (10 or 30 min) were able to extract cholesterol from the outer leaflet of the plasma membrane without inducing the efflux of cholesterol from internal membranes (Chang et al., 2006).

Results obtained in this study demonstrate that in HepG2 cells, under our experimental conditions, both cyclodextrins release cholesterol efficiently being the order of efficiency in accepting cholesterol 2OHpβCD > MβCD. This is in contrast to that observed by Kilsdonk et al. (1995) in L-cells where MβCD induces a release of 90% cholesterol after 8 h of incubation while 2OHpβCD produces an efflux of 50%. Our results show that HePC does not modify the efflux of newly synthesized cholesterol after 6 or 24 h exposure and thence we can deduce that independently of its effect on cholesterol biosynthesis in HepG2 cells, the transport from ER to the plasma membrane is not interfered by this APC.

Cholesterol released from ingested LDL in lysosomes moves to the plasma membrane and afterwards it moves to the ER where it is re-esterified. To examine the effect of HePC on the endocytic pathway, HepG2 cells were loaded with LDL labeled with [^{3}H]CL, and the label was then chased from the plasma membrane by using

MβCD. In a previous work, we found that the treatment with HePC strongly inhibited the re-esterification of LDL-cholesterol in the ER (Carrasco et al., 2008). We have demonstrated now that the APC did not block the [^{3}H]cholesterol transfer from lysosomes to the cell surface. Therefore, we can conclude that the site of action of this APC was not this lysosomal transport. We suggest instead that the ability of this APC to block cholesterol movement from the plasma membrane to the ER is responsible for the inhibition of esterification of cholesterol from LDL observed previously by us (Carrasco et al., 2008).

In order to analyze the molecular mechanism underlying the disruption of intracellular cholesterol homeostasis by HePC we have extended our studies determining the initial effects of this APC on HepG2 cells. In addition, we have studied whether the response of the cells to the APC would be prevented under conditions of increased traffic of cholesterol to the ER. To manage this, we incubated the cells for 60 min in the presence of HePC alone, SMase alone or in combination and determined the extent of CE synthesis. We selected SMase since it has been described that exposure of cells to this enzyme causes the hydrolysis of plasma membrane SM and thereby enhances the amount of plasma-membrane cholesterol fluxing to the ER (Chen et al., 1992; Chatterjee, 1994), this effect being likely related to an alteration in the SM/cholesterol ratio in the plasma membrane. In the present study, we have demonstrated that when HepG2 cells were treated with SMase the synthesis of [^{3}H]cholesteryl oleate was enhanced as a consequence of the increase in the traffic of cholesterol from the plasma membrane to the ER. In addition, and according to that previously reported by us in cells exposed for 6 h to the APC (Jiménez-López et al., 2006; Carrasco et al., 2008), 60 min of HePC treatment also provokes a decrease in the arrival to ER of cholesterol from the plasma membrane and thence a marked inhibition in the esterification of cellular cholesterol occurs.

Plasma-membrane cholesterol is thought to follow at least two pathways to the ER: (1) a vesicular route via endosomes and/or Golgi and (2) a nonvesicular alternative route. When the transport of cholesterol from the plasma membrane to the ER was measured in energy-depleted cells, we found that the synthesis of esterified cholesterol was decreased in an 18% as a result of ATP depletion. Since ATP is required for vesicular transport, this indicates that most of the delivery of cholesterol from the plasma membrane to the ER is nonvesicular. Furthermore, our results indicate that esterification of cholesterol in cells exposed to HePC was similar both in control and energy-depleted cells, demonstrating that this APC only affects the nonvesicular transport.

Interestingly SMase exposure does not prevent at all the inhibition in cholesterol traffic and CE formation produced by HePC. Although nowadays how cholesterol is moved from the plasma membrane to the ER is not yet entirely understood, it has recently been proposed that cholesterol in plasma membrane that is accessible to the ER for cholesterol esterification proceeds from the sterol molecules located in the liquid-disordered domains, i.e. non-raft cholesterol (Simons and Ikonen, 2000; Lange and Steck, 2008). So, the treatment of living cells with SMase degrading sphingolipids in plasma membrane rafts leads to a destruction of rafts moving cholesterol into the non-raft pool, which flows back to the ER. It has also been reported that HePC strongly interacts with cholesterol and other sterols (Ménez et al., 2007; Rakotomanga et al., 2004). Therefore, our results lead us to postulate that HePC, due to its amphiphilic nature, acts on cell membrane, probably in the loosely packed fluid matrix corresponding to non-raft domains, retaining the cholesterol molecules and thence decreasing the influx of the sterol to the ER. This hypothesis can thence explain why in the presence of HePC the cholesterol displaced from rafts to non-raft domains after SMase treatment would be similarly stabilized by HePC disturbing its transport to the ER.

In conclusion, the bulk of our data demonstrate that the main mechanism by which HePC impairs cholesterol homeostasis in HepG2 cells is altering the nonvesicular intracellular transport of cholesterol from the plasma membrane to the ER. The increase in the biosynthesis of cholesterol is accompanied by a marked inhibition of SM synthesis. The ratio SM/cholesterol is known to be crucial in the integrity and functionality of the lipid rafts and consequently the disturbance of this ratio could alter several signaling pathways associated to these microdomains (Langlet et al., 2000; Simons and Toomre, 2000).

Acknowledgements

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4.2. Disruption of cellular cholesterol transport and homeostasis as a novel mechanism of action of membrane-targeted alkylphospholipid analogues

We believe that the first work is an interesting starting point for understanding effects caused by APLs on the biosynthesis and intracellular transport of cholesterol. In the present work we extend our study to four different APLs - HePC, ErPC, edelfosine and perifosine - with aim of finding a possible common mechanism of action. To begin with, we used cristal violet staining to check proliferation in HepG2 cells. All APLs exhibit antiproliferative activity in this cell line. The inhibitory potency of APLs tested shows following pattern: edelfosine ≥ perifosine>ErPC ≥ HePC. These effects were non the result of cytolysis as release of lactate dehydrogenase was similar between control and APL-treated cells. However, cell shape was affected after 24 h APLs exposition as HepG2 cells were rounded shape. All APLs showed an inhibitory effect on cholesterol esterification, from radiolabeled oleate. By tritiated-cholesterol labeling we confirmed that transport of cholesterol from plasma membrane to ER was decreased too in APLs treated cells. This demonstrates that, as reported in the previous work for HePC, all the tested APLs decrease cholesterol transport from plasma membrane to ER.

Furthermore, radioactive acetate incorporated into cholesterol after 24 h APL-treatment was significant higher than in control cells, demonstrating that APLs stimulates cholesterol biosynthesis. Coincidently, Real-Time PCR (qRT-PCR) with arrays for genes related with cholesterol biosynthesis showed that HePC and edelfosine induced HMGCR, HMGCS1, MVD and MVK gene expression. Even more, not only genes but also proteins related with cholesterol processing and uptake as SREBP-2, LDLR or HMGCR were increased too.

In the first work we reported that levels of sphingomyelin and cholesterol, major lipidic constituents of membrane raft microdomains, were disturbed by HePC. To deep on this point, we checked if raft microdomains integrity was affected by APLs. We isolated raft fractions using a free detergent gradient, formed by differential centrifugation, and analyzed in all fractions the lipid composition for control and HePC-treated HepG2 cells. It is remarkable that HePC altered microdomain composition; this is that

cholesterol/sphingomyelin ratio was significantly higher in raft fractions than in untreated ones.

To summarize, in this work we found out a common effect to all APLs that consist of inhibition of cholesterol transport from plasma membrane to ER. This alteration is related with increases in genes and proteins that are involved in cholesterol biosynthesis and uptake. Raft membrane domains are altered too, suggesting that survival signaling pathways raft-dependent could well be affected taking part in the antiproliferative activity of APLs.

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RESEARCH PAPER

Disruption of cellular cholesterol transport and homeostasis as a novel mechanism of action of membrane-targeted alkylphospholipid analogues

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Background and purpose: Alkylphospholipid (APL) analogues constitute a new class of synthetic anti-tumour agents that act directly on cell membranes. We have previously demonstrated that hexadecylphosphocholine (HePC) alters intracellular cholesterol traffic and metabolism in HepG2 cells. We now extended our studies to analyse the effects of other clinically relevant APLs, such as edelfosine, erucylphosphocholine and perifosine on intracellular cholesterol homeostasis.
Experimental approach: Using radiolabelled substrates we determined the effect of APLs on cholesterol metabolism and cholesterol traffic from the plasma membrane to the endoplasmic reticulum (ER). Protein levels and gene expression of the main proteins involved in cholesterol homeostasis were analysed by Western blot and RT-PCR respectively. Membrane raft and non-raft fractions were isolated from HepG2 cells by a detergent-free method.
Key results: All APLs inhibited the transport of cholesterol from the plasma membrane to the ER, which induced a significant cholesterogenic response in HepG2 cells. This response involved an increased gene expression and higher levels of several proteins related to the biosynthesis and the receptor-mediated uptake of cholesterol. Cell exposure to the APL-representative HePC enhanced the content of cholesterol mainly in the membrane raft fractions, compared with the untreated cells.
Conclusions and implications: Membrane-targeted APLs exhibited a novel and common mechanism of action, through disruption of cholesterol homeostasis, which in turn affected specific lipid microdomains of cellular membranes.
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Keywords: alkylphospholipid analogues; cholesterol homeostasis; lipid rafts; HepG2 cells

Abbreviations: APL, alkylphospholipid; CT, CTP : phosphocholine cytidylyltransferase; ER, endoplasmic reticulum; ErPC, erucylphosphocholine; FBS, fetal bovine serum; FDFT1, farnesyl diphosphate farnesyltransferase-1; FDPS, farnesyl diphosphate synthase; HePC, hexadecylphosphocholine; HMGCR, 3-hydroxy-3-methylglutaryl-CoA reductase; HMGCS1, HMG-CoA synthase-1; HRP, horseradish peroxidase; LDH, lactate dehydrogenase; LDLR, low-density lipoprotein receptor; MEM, minimal essential medium; PCR, polymerase chain reaction; SREBP, sterol regulatory element-binding protein

Introduction

The synthetic lipid analogue, hexadecylphosphocholine (HePC) belongs to a new class of anti-tumour agents, which, in contrast to most of the currently used chemotherapeutic drugs, does not target DNA but acts at the cell membrane (van Blitterswijk and Verheij, 2008; Barratt *et al.*, 2009). In addition to its anti-neoplastic activity, HePC is used as a drug for the oral treatment of human cutaneous and visceral leishmaniasis (Soto and Soto, 2006; Rakotomanga *et al.*, 2007) and is also toxic *in vitro* to other protozoan parasites (Seifert *et al.*, 2001; Saraiva *et al.*, 2002; Walochnik *et al.*, 2002).

Because of the hydrophobic nature of its long hydrocarbon chain, HePC may become incorporated into the plasma membrane of cells and resist catabolic degradation. The level of partitioning into membrane lipid bilayers is related to the degree of phospholipid alkyl chain unsaturation and the amount of cholesterol. The affinity of HePC for sterols suggests that it could interact with biological membranes thus interfering with lipid metabolism and lipid-dependent signal transduction (Barratt *et al.*, 2009). Consistent with this suggestion, we found earlier that the alkylphosphocholine HePC interferes with phosphatidylcholine synthesis in human hepatoma HepG2 cells via both CDP-choline (Jiménez-López *et al.*, 2002) and phosphatidylethanolamine methylation pathways (Jiménez-López *et al.*, 2004).

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HePC belongs to a second generation of the synthetic compounds called alkylphospholipid (APL) analogues, the prototype of the first generation being edelfosine, which structurally resembles lysophosphatidylcholine. In an attempt to improve anti-tumour activity with reduced side effects, erucylphosphocholine (ErPC) and perifosine were synthesized. Compared with HePC, ErPC contains a longer chain with a *cis* double bond and perifosine presents a piperidine moiety instead of the choline head group (van Blitterswijk and Verheij, 2008). All of them share structural features and act at cell membranes by interfering with the turnover and synthesis of phospholipids. Thus, they induce apoptosis through inhibition of CTP : phosphocholine cytidylyltransferase (CT), a key enzyme in phosphatidylcholine biosynthesis (van der Luit *et al.*, 2007). These authors reported that, in mouse S49 lymphoma cells, edelfosine and other APL analogues accumulate in detergent-resistant, sphingolipid- and cholesterol-enriched lipid raft domains and are rapidly internalized by clathrin-independent, raft-mediated endocytosis to inhibit CT activity. A cell line made resistant to APLs was incapable of edelfosine internalization via this raft-dependent pathway. APL uptake in KB carcinoma cells, however, appears to be raft-independent and mediated by a yet undefined ATP-driven lipid transporter (Vink *et al.*, 2007b). Particularly, it has been reported by Mollinedo's group that edelfosine treatment induces the formation of membrane raft aggregates containing Fas/CD95 death receptor and the adaptor molecule Fas-associated death domain-containing protein (FADD), which are critical in the triggering of apoptosis induced by edelfosine and other APLs in leukemic cells (Gajate *et al.*, 2009).

Recently, we have established that HePC also alters intracellular cholesterol traffic and metabolism leading to an increased uptake, synthesis and accumulation of cholesterol in the cell (Jiménez-López *et al.*, 2006; Carrasco *et al.*, 2008; Marco *et al.*, 2009). In the present work, we have extended our studies to analyse the effects of a variety of clinically relevant APLs on intracellular cholesterol homeostasis, thus affecting membrane lipid composition and their distribution in raft-non-raft domains. Alterations induced by APLs in the homeostasis of cholesterol provide a novel mechanism of action for membrane-targeted APLs that may well contribute to their anti-tumour activity.

Methods

Cell culture

The human hepatoma HepG2 cell line was from The European Collection of Animal Cell Cultures (Salisbury, UK). Cells were cultured in minimal essential medium (MEM) containing 10% heat-inactivated fetal bovine serum (FBS), supplemented with 2 mM L-glutamine, 1% non-essential amino acids, 100 U·mL^{-1} penicillin and 100 μg·mL^{-1} streptomycin. Cells were grown in a humidified atmosphere with 5% CO_2 at 37°C and were subcultured at a 1:10 ratio once a week. Cells were plated on tissue-culture dishes (Nunc™, from LabClinics SA, Barcelona, Spain) at a density of 5×10^4 cells·cm^{-2} and maintained in culture medium for 3 days before being used in experimental radiolabelling assays at approximately 70% confluence.

Assays for cell viability and proliferation

Cells were seeded onto 24-well plates (25 000 cells·well^{-1}) and maintained in MEM containing 10% FBS for 24 h. Then, the culture medium was replaced with fresh medium/10% FBS and the cells were incubated for 48 h in the absence or presence of APLs (up to 75 μM) before analyses. The antiproliferative effect of the different APL analogues on HepG2 cells was assessed by the crystal violet staining assay using a cell number-based standard curve. The indexes of cytotoxicity used in this study were the measurements of lactate dehydrogenase (LDH) leakage and the change in cell morphology observed under the inverted microscope.

Metabolic labelling assays

The synthesis of cholesterol was determined by measuring the incorporation of radioactive exogenous acetate into cellular sterols. Log-phase HepG2 cells were incubated in medium containing 10% FBS either without (control) or with 25 μM HePC, edelfosine, ErPC or perifosine for 24 h. [1-^{14}C]acetate (87 μM, 58 Ci·mol^{-1}) was added during the last 6 h of incubation. After labelling, the medium was removed and the cells were washed twice with ice-cold PBS before being harvested by scraping into PBS. The lipids were extracted from the cells following the procedure of Bligh and Dyer (1959). Cholesterol was separated by TLC using a mixture of hexane/diethyl ether/acetic acid (80/20/2, v/v/v) as solvent.

To analyse the synthesis of cholesteryl esters, HepG2 cells were incubated in MEM/10% FBS without or with the APLs (25 μM) for 1 h at 37°C in the presence of [9,10 (n)-^{3}H]oleate (100 μM, 34 Ci·mol^{-1}). Cholesteryl [^{3}H]oleate was isolated from the cells by TLC using hexane/diethyl ether/acetic acid (80/20/2, v/v/v) as solvent.

Radiometric measurements of scraped lipid spots, rendered visible by exposure to iodine vapour, were made by liquid scintillation counting.

Trafficking of cholesterol from the plasma membrane to the endoplasmic reticulum

An appropriate way to measure cholesterol transport from the plasma membrane to the endoplasmic reticulum (ER) is to determine the degree of esterification of radiolabelled cholesterol previously incorporated into the plasma membrane (Lange and Steck, 1997; Marco *et al.*, 2009). Thus, HepG2 cells were incubated with 2 μCi of [7(n)^3-^{3}H]cholesterol (dissolved in 2-propanol) for 1 h at 37°C to label the plasma membrane cholesterol. To remove any unincorporated radioactivity, the cells were washed twice with PBS containing 0.5% BSA prewarmed to 37°C. The cells were then incubated at 37°C in MEM for 1 h in the absence or presence of 25 μM HePC, edelfosine, ErPC or perifosine. Then, the medium was removed and the lipids were extracted and analysed as described above.

Immunoblot analysis

HepG2 cells growing in log-phase were incubated with MEM containing 10% FBS in the absence (PBS as the vehicle) or presence of 25 μM HePC, edelfosine, ErPC or perifosine for

three different periods (6, 24 or 48 h). The cells were washed twice and scraped into ice-cold PBS (pH 7.4), and centrifuged at 100× *g* for 10 min at 4°C. Cell pellets were suspended in 0.3 mL ice-cold lysis buffer consisting of 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1% Triton X-100 and a protease inhibitor cocktail from Sigma (Madrid, Spain), and incubated on ice for 30 min with occasional shaking. Cell lysates were centrifuged at 10 000× *g* for 15 min at 4°C, and supernatants were stored at –80°C until use; an aliquot was taken to determine protein concentration. Equal amounts of lysate protein (30 μg) were separated by 10% SDS-PAGE and transferred onto polyvinylidene difluoride membranes. Prestained broad-range protein molecular-mass markers were used during electrophoresis. Membranes were blocked in PBS containing 5% non-fat dry milk and 0.05% Tween-20, and then probed with the indicated polyclonal anti-human primary Ig (1:1000) in blocking solution for 1 h. After several washes in PBS containing 0.05% Tween-20, the membranes were incubated with the corresponding horseradish peroxidase (HRP)-conjugated IgG (1:5000) as secondary antibody for 1 h. Immunoreactive proteins were detected by autoradiography using a chemiluminescent HRP substrate and exposure to Konica Minolta X-ray film (Tokyo, Japan). Following incubation with an antibody-stripping solution consisting of 60 mM Tris-HCl (pH 6.8), 100 mM β-mercaptoethanol and 2% SDS for 30 min at 60°C, blots were probed with rabbit polyclonal anti-human actin Ig (1:1000) to monitor the loading and transfer of the blotted samples. Densitometric analysis was carried out using the ImageJ gel-digitizing software from the National Institutes of Health (Bethesda, MD, USA).

Quantification of mRNA using real-time polymerase chain reaction array system

Exponentially growing HepG2 cells were incubated with MEM containing 10% FBS in the absence (PBS as the vehicle) or presence of 25 μM HePC or edelfosine for three different periods (6, 24 or 48 h). Total RNA was isolated using the RNeasy Mini kit (Qiagen, Iberia SL, Madrid, Spain) and reverse-transcribed into cDNA using the RT2 First Strand kit from SABiosciences (Frederick, MD, USA), which includes both oligo-dT and random hexamer primers. SYBR Green real-time polymerase chain reaction (PCR) was performed on a Chromo4 real-time PCR detector (Bio-Rad Laboratories SA, Madrid, Spain) using the human lipoprotein signalling and cholesterol metabolism RT2 profiler™ PCR array from SABiosciences, which profiles the expression of a focused panel of genes related to lipoprotein transport and cholesterol metabolism. The array also includes sets of replicate control wells that specifically detect non-transcribed genomic DNA contamination, the efficiency of reverse transcription or the efficiency of positive PCR reactions, and specific primer sets for the amplification of five housekeeping gene transcripts: β-2-microglobulin, hypoxanthine phosphoribosyltransferase 1, ribosomal protein L13a, glyceraldehyde-3-phosphate dehydrogenase and β-actin. Reactions were run in duplicate from three independent biological samples for each condition. As a quality control, a dissociation (melting) curve was acquired immediately after the PCR cycling programme. The relative expression ratio was determined from the averaged threshold cycle values, that is, $\Delta\Delta C_T$-based fold change calculations, using an integrated web-based software package from SABiosciences for the PCR array data analysis.

Detergent-free isolation of membrane fractions from HepG2 cells

Membrane raft and non-raft fractions were isolated from HepG2 cells by the detergent-free method of Macdonald and Pike (2005) with minor modifications. Briefly, the cells were washed and collected into a base buffer consisting of 20 mM Tris-HCl (pH 7.8), 250 mM sucrose, 1 mM $CaCl_2$ and 1 mM $MgCl_2$, supplemented with a protease inhibitor cocktail (Sigma). Cells were incubated on ice and lysed by successive passages through a 22 g × 3″ needle 10 times, then a 25 g × 3″ needle 20 times and, finally, a 30 g × 0.32″ needle 10 times, and lysates were centrifuged at 1000× *g* for 10 min at 4°C. The post-nuclear supernatant was collected, and the process was repeated on the pellet. Both resulting supernatants were combined and immediately subjected to centrifugation in a 12 mL step density gradient of 3–25% OptiPrep™ [a 60% (w/v) iodixanol stock solution in water] for 90 min at 52 000× *g* using a SW-41 rotor in a Beckman ultracentrifuge. Gradients were fractionated into 0.67 mL aliquots collected from the top of the gradient; the fractions were stored at –80°C until use.

Distribution of the raft-marker GM1 ganglioside was examined by dot-blot immunodetection using the ability of HRP-conjugated cholera toxin B subunit to bind GM1 in the samples transferred individually onto a polyvinylidene difluoride membrane.

Other analyses

Lipids were isolated from both raft and non-raft fractions of controls and HePC-treated HepG2 cells. Total cholesterol content was measured by an enzymatic colorimetric kit from LabKit (Madrid, Spain). The levels of sphingomyelin (SM) were quantified by Bartlett's method (1959). Protein concentrations were determined by the Bradford's method (1976) using BSA as standard.

Statistics

The results were expressed as means ± SEM. One-way ANOVA with subsequent *post hoc* comparisons by Scheffe's test was carried out (SPSS 13.0). *P*-values < 0.05 were considered to be statistically significant.

Materials

Radiolabelled compounds were supplied by American Radiolabeled Chemicals (St Louis, MO, USA). FBS was from The Cell Culture Company (Pasching, Austria). MEM and TLC plates were from Sigma-Aldrich (Madrid, Spain). HePC was supplied by Cayman Chemical (Ann Arbor, MI, USA), edelfosine by Calbiochem (Nottingham, UK), ErPC by Alexis Biochemicals (Exeter, UK) and perifosine by Selleck Chemical (London, ON, Canada). Polyclonal anti-human primary antibodies and HRP-conjugated secondary IgGs were from Santa Cruz Biotechnology (Santa Cruz, CA, USA), except anti-human 3-hydroxy-3-methylglutaryl-CoA reductase (HMGCR) primary

Ig, which was acquired from Upstate (Lake Placid, NY, USA). HRP-conjugated cholera toxin B subunit was from Life Technologies Corp. (Carlsbad, CA, USA). The enhanced chemiluminescence detection system was from Millipore (Billerica, MA, USA). All other reagents were of analytical grade. Drug/molecular target nomenclature follows Alexander *et al.* (2009).

Results

Effects of alkylphospholipids on cell proliferation and viability in HepG2 cells

We first analysed the effect of various APLs – HePC, edelfosine, ErPC and perifosine – on cell proliferation. To do this, HepG2 cells were treated with increasing concentrations of APLs for up to 48 h, and cell number in control and APL-treated cells was assessed by crystal violet staining. All the tested APLs displayed growth inhibitory effects in a dose-dependent manner in the HepG2 cells, which became more pronounced as treatment time increased. However, it is important to note that the different APLs exhibited clear differences in their inhibitory potency. So, as shown in Figure 1 all the APLs similarly inhibited the growth rate up to 25 μM. However, at amounts higher than 25 μM, edelfosine and perifosine decreased the proliferation rate to a greater extent than either ErPC or HePC did after 48 h of cell exposure. After 48 h treatment, the values of IC_{50} derived from the growth inhibition curves were approximately 40 μM for edelfosine and perifosine, while the other two APLs needed double the concentration to produce the same inhibitory effect. The inhibitory potency of the four tested APLs shows the following pattern: edelfosine ≥ perifosine > ErPC > HePC.

It is important to note that the decrease in cell number observed after 48 h of APL treatment was not related to any acute cytotoxicity produced by plasma membrane leakage, as we did not detect LDH activity released into the culture medium after any of the treatments assayed up to 50 μM; slight toxicity was only apparent after prolonged treatment with 75 μM edelfosine or perifosine (data not shown).

Concomitantly, morphological changes were also found to be induced in cells treated with the different APLs such as condensation and rounding, traits of cell damage and initiation of apoptosis (Figure 2). Again, edelfosine and perifosine treatments affected HepG2 cells more profoundly than HePC and ErPC.

Effects of alkylphospholipids on cholesterol traffic and homeostasis

Previous studies in our laboratory have shown that treatment of HepG2 and Vero cells with HePC significantly modulates cholesterol metabolism and hence intracellular cholesterol levels are enhanced (Jiménez-López *et al.*, 2006). The increase induced by HePC in cholesterogenic activity was found both in the presence or absence of exogenous LDL-cholesterol (Carrasco *et al.*, 2008). To determine whether other APLs show the same effect, we determined cholesterol biosynthesis by the incorporation of radiolabelled acetate into cholesterol in HepG2 cells incubated with different APLs. Interestingly, similar to HePC, edelfosine, ErPC and perifosine all significantly stimulated *de novo* biosynthesis of cholesterol, leading to an increase in cholesterogenic activity. Edelfosine (90% activation) was the most potent followed by ErPC, HePC and perifosine, amounting to 80%, 60% and 40% activation respectively (Figure 3).

Recently, we described that exposure of HepG2 cells to 50 μM HePC for 1 h causes a drastic reduction in the synthesis of [^{3}H]cholesteryl oleate from radiolabelled oleate (Marco *et al.*, 2009). To test whether it occurs also for other APLs,

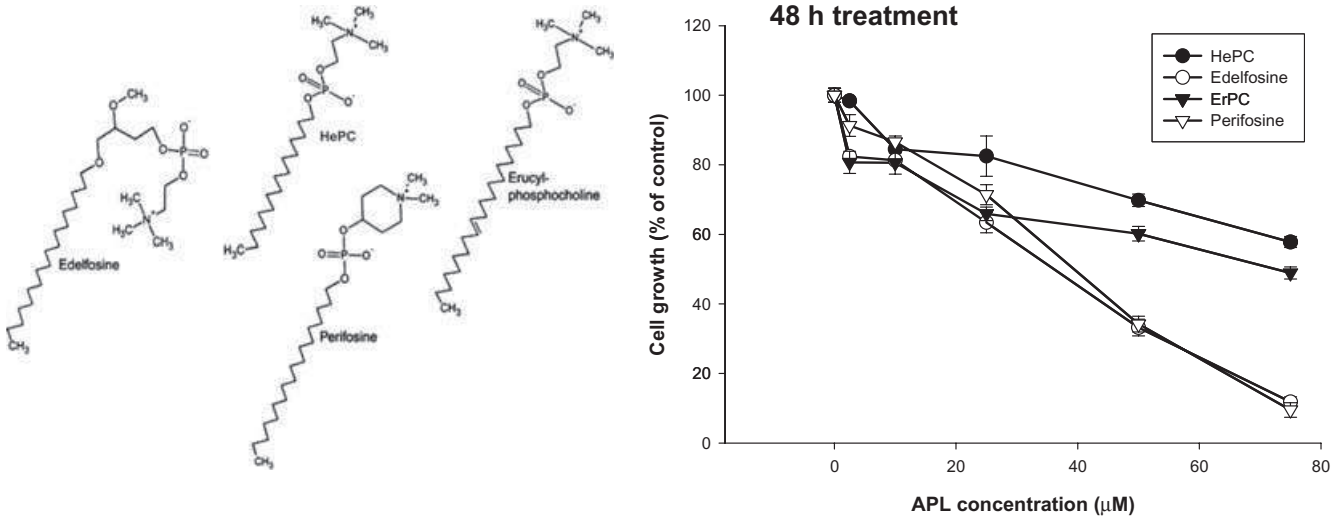


Figure 1 Inhibition curves in HepG2 cells exposed to different concentrations of alkylphospholipids. Cells growing in log-phase were incubated with MEM/10% FBS in the absence or presence of different concentrations of APLs for 48 h. Cell number was determined by the crystal violet staining assay and expressed as percentage of control (no addition) cells. Results represent the mean ± SEM of two independent experiments conducted in triplicate. Chemical structures of synthetic APLs used in this study are shown on the left of the Figure (adapted from Vink *et al.*, 2007a). APL, alkylphospholipid; ErPC, erucylphosphocholine; FBS, fetal bovine serum; HePC, hexadecylphosphocholine; MEM, minimal essential medium.

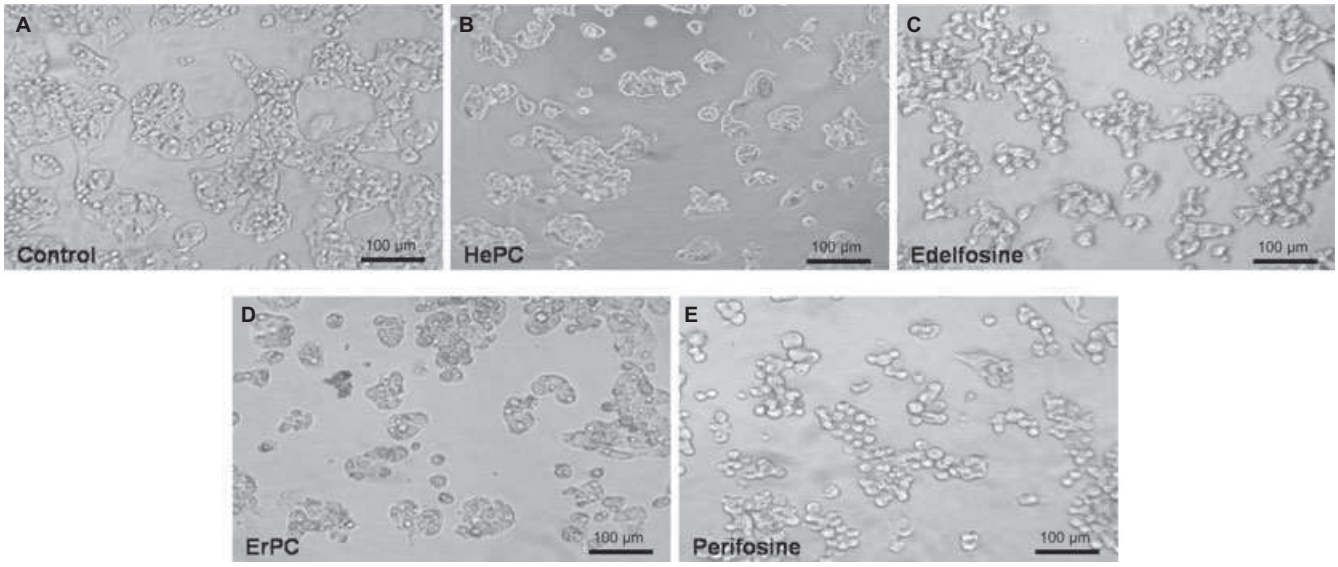


Figure 2 Alkylphospholipid-induced morphological changes in HepG2 cells. Cell morphology was examined with an inverted microscope (20× original magnification). The morphology of HepG2 cells incubated with MEM/10% FBS is shown in the absence of any addition (control, A), or in the presence of 25 μM of HePC (B), edelfosine (C), ErPC (D) or perifosine (E) for 24 h. ErPC, erucylphosphocholine; FBS, fetal bovine serum; HePC, hexadecylphosphocholine; MEM, minimal essential medium.

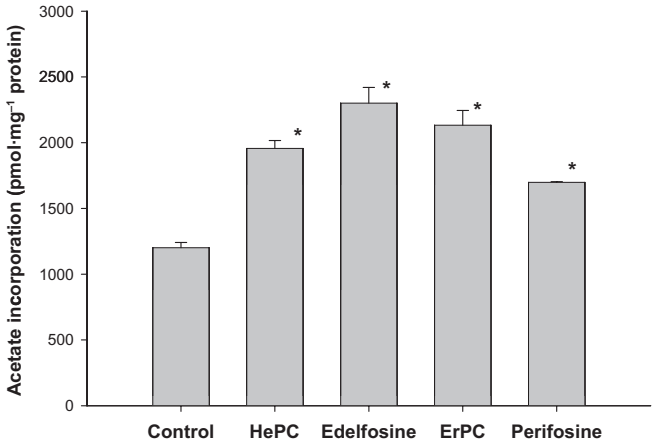


Figure 3 Effect of alkylphospholipids on the biosynthesis of cholesterol. HepG2 cells growing in log-phase were treated with 25 μM HePC, edelfosine, ErPC or perifosine for 24 h in MEM/10% FBS or without any additions (control). [1-^{14}C]acetate (3.4 μM, 60 Ci·mol^{-1}) was added during the last 6 h incubation period. The incorporation of acetate into cholesterol was determined. Results are expressed as pmol of acetate incorporated per mg of cell protein and represent the mean ± SEM of two independent experiments conducted in triplicate. *P < 0.001 when compared with control values. ErPC, erucylphosphocholine; FBS, fetal bovine serum; HePC, hexadecylphosphocholine; MEM, minimal essential medium.

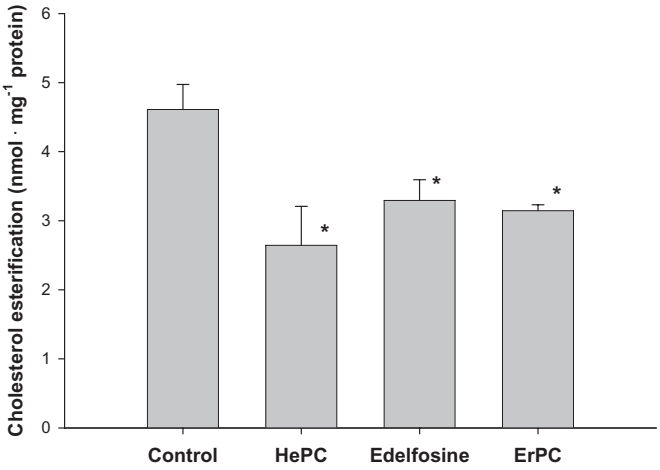


Figure 4 Effect of alkylphospholipids on the esterification of cholesterol. Log-phase HepG2 cells were incubated with [9,10 (n)-^{3}H]oleate (100 μM, 34 Ci·mol^{-1}) for 1 h in serum-free medium either with 25 μM HePC, edelfosine or ErPC or without any additions (control). The incorporation of oleate into cholesteryl oleate was determined. The results are expressed as nmol of oleate incorporated per mg of cell protein and represent the mean ± SEM of two independent experiments conducted in triplicate. *P < 0.02 when compared with control values. ErPC, erucylphosphocholine; HePC, hexadecylphosphocholine.

HepG2 cells were incubated with [9,10 (n)-^{3}H]oleate in the absence or presence of the APLs and the synthesis of [^{3}H]cholesteryl oleate was measured. The APLs were found to inhibit esterified cholesterol synthesis, HePC being the most potent, followed by edelfosine and ErPC (Figure 4).

Previously, we demonstrated that 50 μM HePC blocked the esterification of plasma membrane cholesterol, confirming that the inhibition caused by HePC in the esterification of the intracellular cholesterol pool is produced by a disruption of its movement from the plasma membrane to the ER (Marco *et al.*, 2009). We have used the esterification of plasma membrane cholesterol as a marker of cholesterol movement from the cell surface to the ER, the site of acyl-CoA : cholesterol acyltransferase activity (Marco *et al.*, 2009). Our results indicated that edelfosine, ErPC and perifosine produce a remarkable decrease of 80% in cholesterol esterification. This effect was slightly higher than that obtained with the prototypical APL, HePC, around 70% (Figure 5).

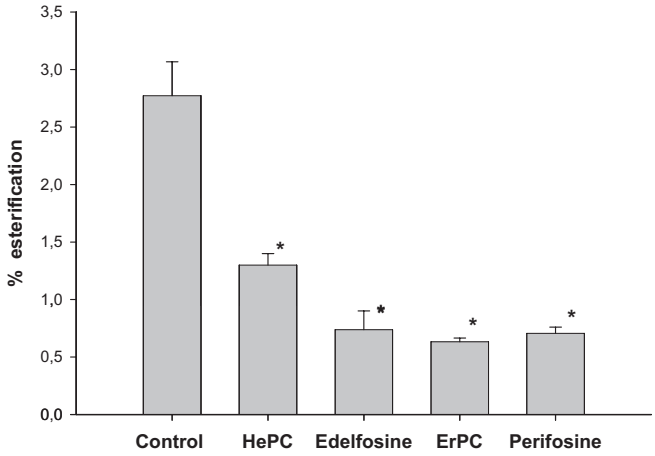


Figure 5 Effect of alkylphospholipids on trafficking of cholesterol from the plasma membrane to the endoplasmic reticulum. Plasma membrane cholesterol in the HepG2 cells was labelled during incubation with 2 μCi [7(n)^3-^{3}H]cholesterol for 1 h in serum-free medium. Then, cells were incubated for 1 h with 25 μM HePC, edelfosine, ErPC or perifosine or without any additions (control). The fraction of plasma membrane cholesterol that was esterified is expressed in terms of the percentage of esterification of the total labelled cholesterol and represents the mean ± SEM of two independent experiments conducted in triplicate. *$P < 0.01$ when compared with control values. ErPC, erucylphosphocholine; HePC, hexadecylphosphocholine.

Effect of alkylphospholipids on the expression of proteins involved in cholesterol homeostasis

Cholesterogenesis is known to be transiently induced by the translocation of the sterol regulatory element-binding protein 2 (SREBP2) transcription factor from the ER membrane (125 kDa precursor form) to the nucleus (70 kDa mature form) (Horton *et al.*, 2003). To validate a role for SREBP2 in the actions of APLs, we analysed by immunoblotting the effect caused by APLs on the expression of SREBP2, and known key SREBP2 targets such as HMGCR and low-density lipoprotein receptor (LDLR) (Horton *et al.*, 2003; Bennett *et al.*, 2008) in the HepG2 cells. Incubation of cells with the different APLs produced a time-dependent increase of the mature form of SREBP2 in the assayed cell lysates, along with increased protein expression of its targets, HMGCR and LDLR (Figure 6). High expression levels of SREBP2 were observed after 24 h treatment with HePC, edelfosine, ErPC or perifosine when compared with basal levels in untreated cells, whereas the membrane-bound precursor form decreased in parallel with the appearance of the released active form (Figure 6 and data not shown).

Exposure to HePC and edelfosine stimulates the expression of genes related to cholesterol homeostasis in HepG2 cells

Bearing in mind the results presented above, we proceeded to analyse how exposure to APL analogues modulated the pathway of cholesterol biosynthesis at the transcriptional level in HepG2 cells. For this purpose we selected HePC and edelfosine as representatives of this family of compounds.

Alterations in gene expression in response to treatment with HePC or edelfosine for 6, 24 or 48 h were analysed using the RT2 Profiler™ PCR Array System. Remarkably, Figure 7A shows that exposure of HepG2 cells to 25 μM HePC increased, in a time-dependent manner, the mRNA transcript levels of transcriptionally regulated, cholesterol-synthesizing enzymes such as HMG-CoA synthase (HMGCS1), HMGCR, farnesyl diphosphate synthase (FDPS) and farnesyl diphosphate farnesyltransferase-1 (FDFT1, also known as squalene synthase), which are rate-limiting enzymes of the cholesterol biosynthetic pathway (Sato and Takano, 1995). Similar to HePC, exposure of cells to 25 μM edelfosine also stimulated the main cholesterogenic genes (Figure 7B).

The expression of another set of genes related to lipid homeostasis was analysed in the HepG2 cells exposed to HePC or edelfosine for several periods of time (Figure 7C and D). In mammalian cells, cholesterol-related genes tend to be more strongly activated by overexpression of nuclear SREBP2 form whereas genes that participate in the synthesis of fatty acids are more strongly affected by overexpression of the nuclear SREBP1 form (Horton *et al.*, 2003). Incubation of cells with 25 μM HePC or edelfosine for 24 h stimulated transcription of SREBP2, that is, SREBP2 mRNA expression increased by 1.35-fold as compared with the untreated cells. Moreover, expression of the SREBP2-responsive LDLR gene was enhanced by both agents (Figure 7C and D), confirming earlier reported data obtained using TaqMan probes designed for amplification of LDLR (Carrasco *et al.*, 2008). These results were closely correlated with the observed increases in the content of the mature SREBP2, HMGCR and LDLR proteins after APL treatment (Figure 6). Gene expression of the SREBP1 isoform, however, did not increase when the HepG2 cells were cultured in the presence of HePC or edelfosine (Figure 7C and D).

Effect of HePC on lipid composition of membrane fractions in HepG2 cells

In order to analyse possible interference by HePC with specific membrane domains, raft microdomains were isolated from HepG2 cells by a cell disruption procedure that avoids detergent or sonication, that is, the method of Macdonald and Pike (2005) with minor modifications. After cell disruption, whole cell lysates were passed through several syringes as described in the *Methods* section, and membrane fractions displaying distinct lipid–protein composition were obtained by density gradient centrifugation. The gradients were fractionated into 18 fractions and analysed for the distribution of several membrane markers (Figure 8). Fractions were characterized by Western blot analysis of protein markers for membrane raft (flotillin-1) or non-raft (clathrin heavy-chain) regions, dot-blot determination of raft-linked GM1 and measuring protein concentration and levels of cholesterol. As shown in Figure 8, lower-density regions of the gradient with relatively little protein (fractions 1–5) were highly enriched in cholesterol, as well as the raft markers GM1 and flotillin-1. Higher-density fractions (12–18) at the bottom of the gradient showed opposite, non-raft characteristics, that is, lower cholesterol with high protein levels, practically in the absence of raft markers but with a significant appearance of the non-raft marker clathrin heavy-chain. Fractions 6–11 constitute an intermediate fraction probably made up by a mixture of raft and non-raft domains.

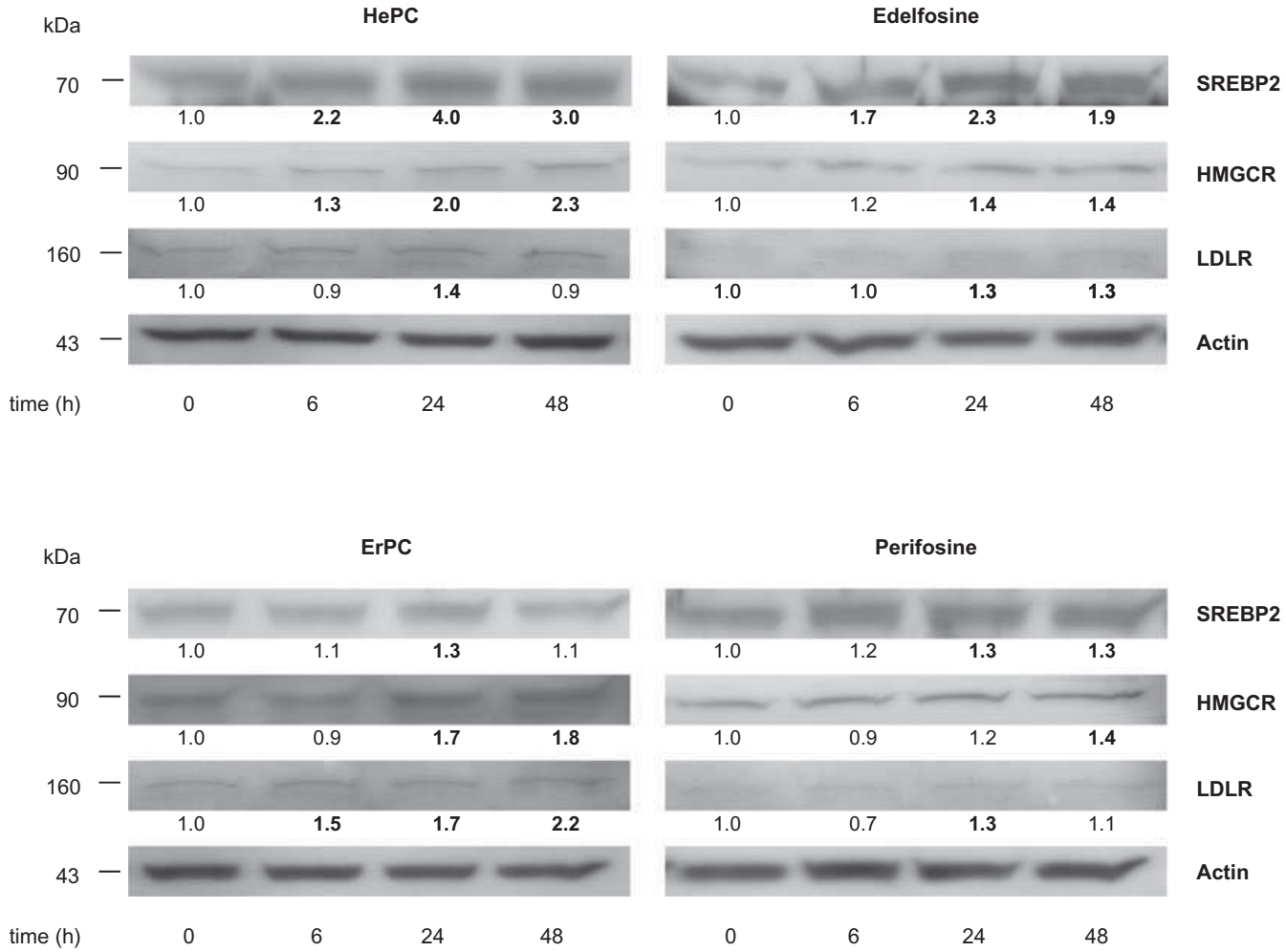


Figure 6 Effect of alkylphospholipids on the expression of proteins involved in the homeostasis of cholesterol. HepG2 cells were incubated with MEM/10% FBS without any additions (control) or containing 25 μM HePC, edelfosine, ErPC or perifosine for 6, 24 or 48 h. Cell lysate samples collected at selected times were analysed by immunoblotting to determine the content of mature SREBP2 form, HMGCR, LDLR and β-actin (loading control). The different bands were scanned and arbitrary units were assigned by densitometric analysis. SREBP2, HMGCR and LDL protein levels in the samples were normalized to their respective β-actin level and expressed as *x*-fold increase compared with the corresponding control ratio (1.0). The figure shows a representative experiment repeated twice. ErPC, erucylphosphocholine; FBS, fetal bovine serum; HePC, hexadecylphosphocholine; HMGCR, 3-hydroxy-3-methylglutaryl-CoA reductase; LDLR, low-density lipoprotein receptor; MEM, minimal essential medium; SREBP, sterol regulatory element-binding protein.

Although the cholesterol and sphingolipid enrichment of lipid rafts relative to whole plasma membrane is well established, relatively few studies have directly quantified lipid composition of membrane raft fractions isolated by detergent-free methods. Our results confirm the higher content of cholesterol and sphingomyelin in fractions 1–5 (rafts) versus the 6–11 (intermediate) and 12–18 (non-rafts) (Table 1). If only the lightest fractions of the Optiprep gradient are pooled, a relatively clean cholesterol- and sphingomyelin-enriched raft fraction can be obtained. Thus, further studies were carried out only with the raft and non-raft fractions.

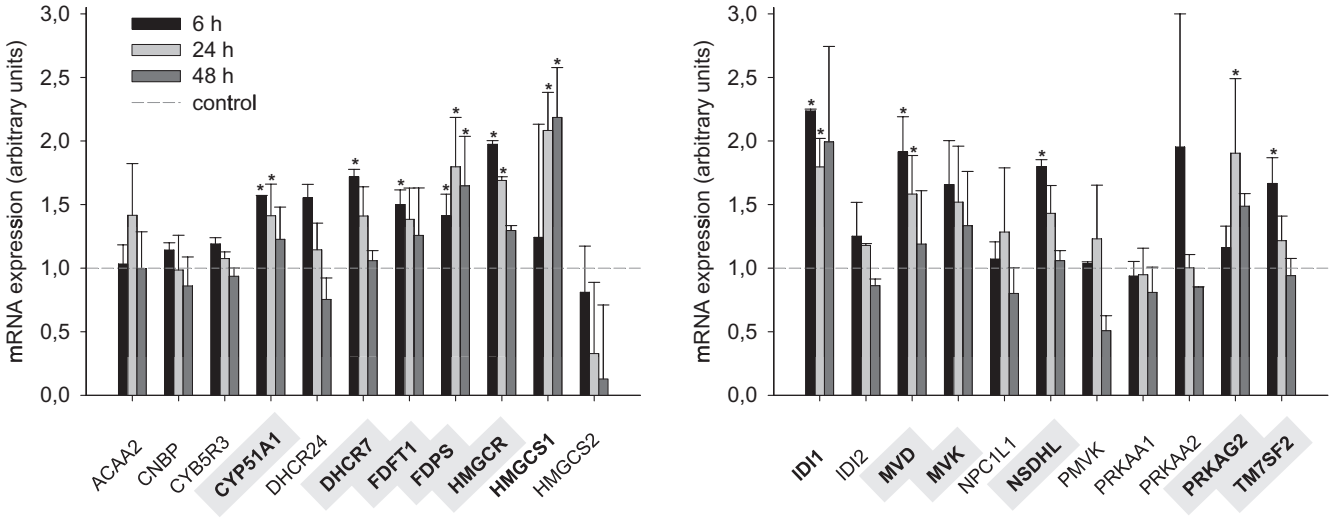
To investigate the effects of HePC treatment on plasma membrane lipid distribution, we analysed the lipid composition of both raft and non-raft membrane fractions after cell incubation with 25 μM HePC for 1, 12 and 24 h. We assessed GM1 localization after HePC treatment and did not detect any change in GM1 distribution along the different-density fractions (Figure 9).

Results in Figure 9 show a marked increase in the content of cholesterol in rafts isolated from HepG2 cells treated with HePC for 24 h, when compared with the control, while only a little increase was found in the non-raft fraction. The content of sphingomyelin was not significantly altered after the same period of treatment. As a consequence of these changes, the cholesterol/sphingomyelin ratio was clearly increased, in a time-dependent manner, by HePC treatment, mainly in the raft-enriched fraction (Figure 9).

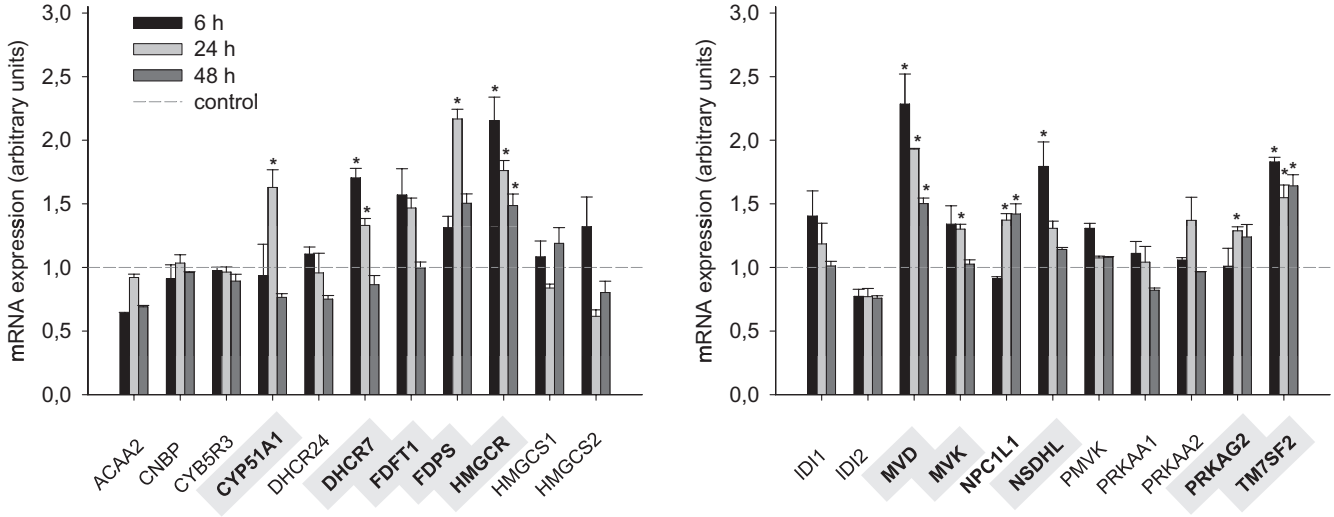
Discussion

It is widely accepted that synthetic APL derivatives have antitumour activity although their anti-proliferative action strongly depends on the cell type and the APL under study (van Blitterswijk and Verheij, 2008). Thus, IC_{50} values from 1 μM up to more than 150 μM have been reported in different cell lines for the different APLs. The IC_{50} values obtained in

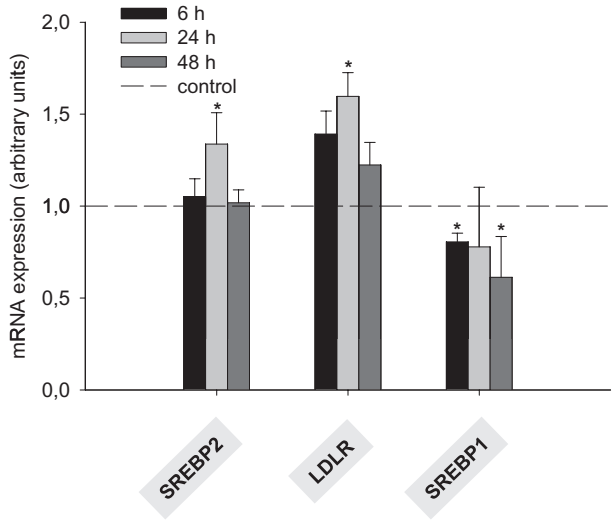
A HePC



B Edelfosine



C HePC



D Edelfosine

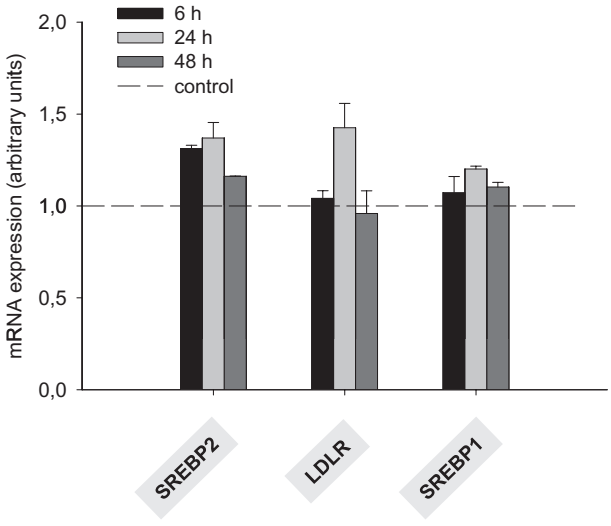


Figure 7 Effect of hexadecylphosphocholine and edelfosine on the expression of genes related to the homeostasis of cholesterol. HepG2 cells were incubated with MEM (minimal essential medium)/10% FBS (fetal bovine serum) in the absence (control) or presence of 25 μM HePC (hexadecylphosphocholine) or edelfosine for three different periods of time (6, 24 or 48 h). The levels of mRNA expression, relative to five housekeeping genes, are shown for a variety of transcriptionally regulated genes involved in cholesterol biosynthesis (A and B), and other genes involved in cholesterol homeostasis (C and D), using real-time PCR. Gene description: ACAA2 (acetyl-CoA acyltransferase 2); CNBP (CCHC-type zinc finger, nucleic acid binding protein); CYP5R3 (cytochrome b5 reductase 3); CYP51A1 (cytochrome P450, family 51, subfamily A, polypeptide 1); DHCR24 (24-dehydrocholesterol reductase); DHCR7 (7-dehydrocholesterol reductase); FDFT1 (farnesyl-diphosphate farnesyltransferase 1); FDPS (farnesyl diphosphate synthase); HMGCR (3-hydroxy-3-methylglutaryl-CoA reductase); HMGCS1 [3-hydroxy-3-methylglutaryl-CoA synthase 1 (soluble)]; HMGCS2 [3-hydroxy-3-methylglutaryl-CoA synthase 2 (mitochondrial)]; IDI1 (isopentenyl-diphosphate δ-isomerase 1); IDI2 (isopentenyl-diphosphate δ-isomerase 2); LDLR (low-density lipoprotein receptor); MVD [mevalonate (diphospho) decarboxylase]; MVK (mevalonate kinase); NPC1L1 [NPC1 (Niemann-Pick disease, type C1, gene)-like 1]; NSDHL [NAD(P) dependent steroid dehydrogenase-like]; PMVK (phosphomevalonate kinase); PRKAA1 (protein kinase, AMP-activated, α1 catalytic subunit); PRKAA2 (protein kinase, AMP-activated, α2 catalytic subunit); PRKAG2 (protein kinase, AMP-activated, γ2 non-catalytic subunit); SREBP1 (sterol regulatory element-binding protein 1, transcription factor); SREBP2 (sterol regulatory element-binding protein 2, transcription factor); TM7SF2 (transmembrane 7 superfamily member 2). *$P < 0.05$ when compared with control values.

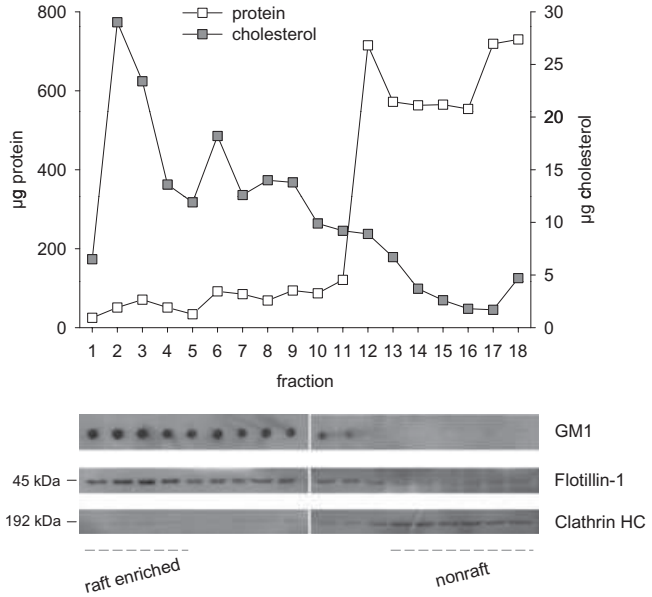


Figure 8 Application of a detergent-free raft isolation procedure to HepG2 cells: distribution of several markers across an Optiprep density gradient. Protein and cholesterol contents were determined in each gradient fraction. Raft-associated GM1 ganglioside was assayed by dot blotting (4 μL sample volumes) using the horseradish peroxidase-conjugated cholera toxin B subunit. A 25 μL aliquot of each gradient fraction was analysed by SDS-PAGE followed by Western blotting for the protein markers flotillin-1 (rafts) and clathrin heavy-chain (clathrin HC; non-rafts).

Table 1 Lipid composition of membrane raft, intermediate and non-raft fractions isolated from control HepG2 cells

nmol of lipid/pooled fraction		
Fractions	*Cholesterol*	*Sphingomyelin*
1–5 (rafts)	1530	2.17
6–11 (intermediate)	570	1.87
12–18 (non-rafts)	390	0.53

our study, related to the anti-proliferative activity exhibited by APLs in hepatoma HepG2 cells, agree well with those previously reported by other authors in several cell lines. For example, Jendrossek *et al*. (2002) have reported that in the U87 MG and C6 cell lines, ErPC concentrations of 80–90 μM decrease cell number up to 50% of controls after 72 h of treatment, while HePC needed concentrations higher than 150 μM to achieve the same anti-proliferative action. Similarly, IC_{50} values for perifosine after 24 h were higher than 40 μM in Ishikawa and HEC 1A human endothelial cancer cell lines (Engel *et al.*, 2008). Few reports have explored the effects of several APLs on the same cell line to compare their inhibitory potency. Wieder *et al*. (1995) comparing edelfosine and HePC in MDCK cells concluded that HePC required twice the concentration, compared with edelfosine, to produce the same inhibitory effect on cell growth. Results in our study demonstrated that in the hepatoma HepG2 cell line, HePC had the lowest anti-proliferative activity while edelfosine presented higher potency in inhibiting cell growth. Due to their chemical structure, APLs easily insert into the membrane thereby causing detergent-like cell lysis at high concentrations. Nevertheless, in our study, the anti-proliferative action of the four APLs was not related to cytolysis, as prolonged incubation of cells with doses up to 50 μM did not cause a significant release of LDH from the cells into the culture medium.

Of particular interest in our previous studies were the effects caused by HePC upon cholesterol metabolism. We have previously reported that long-term HePC treatment of HepG2 cells causes a marked enhancement in cholesterol synthesis (Jiménez-López *et al.*, 2006) that has been related to an impairment in the arrival of cholesterol at the ER (Carrasco *et al.*, 2008; Marco *et al.*, 2009). To our knowledge, there are no data concerning the effects of other APLs on the homeostasis of cholesterol and thus results presented in this paper demonstrate for the first time that, as observed for HePC, other APLs such as edelfosine, ErPC and perifosine increase the *de novo* synthesis and uptake of cholesterol, and inhibit cholesterol esterification, as a consequence of an impaired cholesterol transport from the plasma membrane to the ER in the HepG2 cells. In summary, our results demonstrate that all the APLs assayed affected cell growth and morphology similarly and also altered cholesterol traffic and homeostasis. It must be however stated that, among the four APLs tested, edelfosine exerted the greatest effects on the different parameters measured in HepG2 cells.

Presumably, in the presence of APL, the translocated SREBP2 remained active so as to stimulate gene expression of potential targets in the HepG2 cells. Thus, as a likely

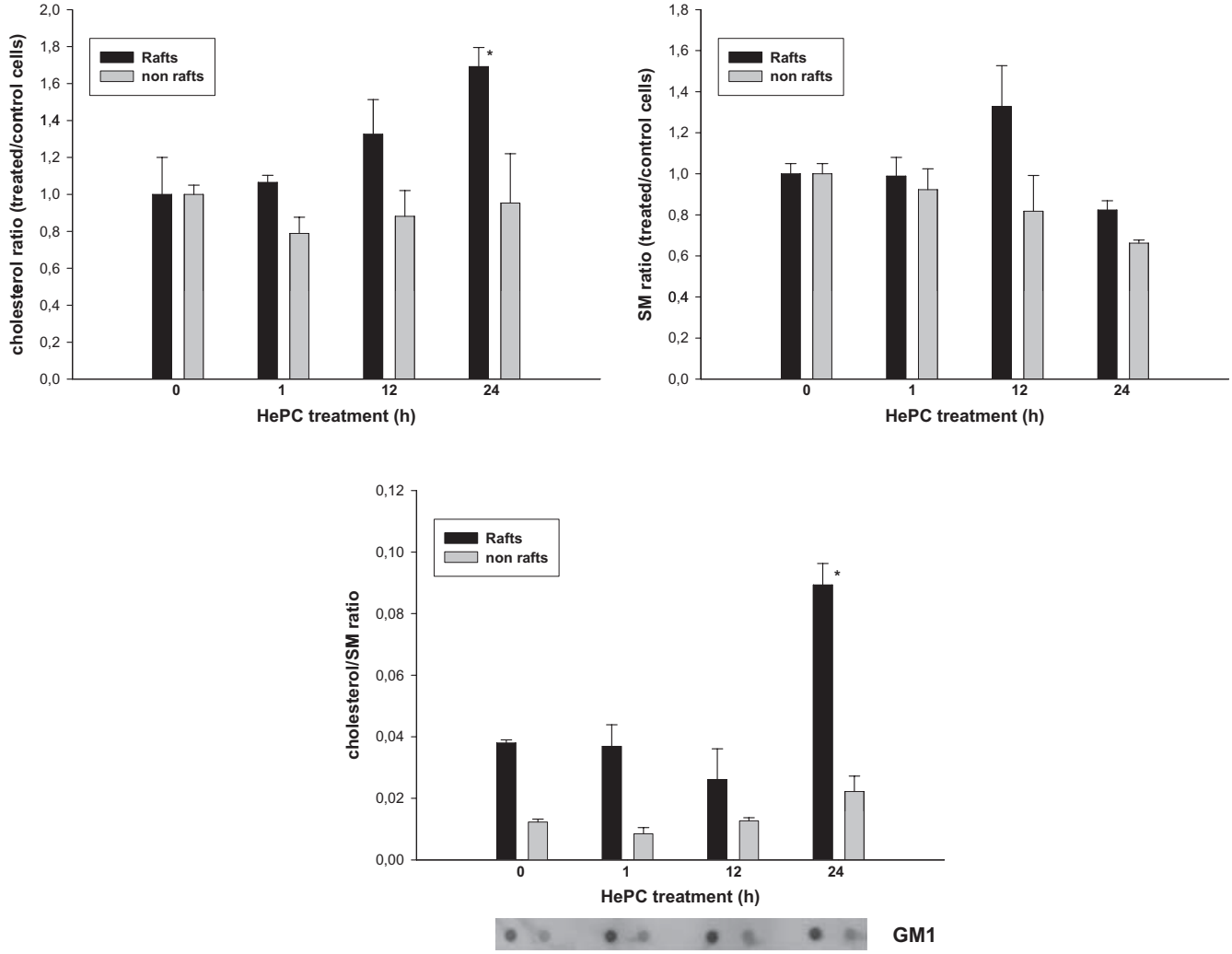


Figure 9 Effect of hexadecylphosphocholine on lipid composition of membrane fractions in HepG2 cells. Cholesterol and sphingomyelin (SM) levels were determined in both raft and non-raft membrane fractions after cell incubation with 25 μM hexadecylphosphocholine (HePC) for 1, 12 and 24 h. Results represent the mean ± SEM of two independent experiments conducted in triplicate. *$P < 0.005$ when compared with control values.

consequence of the APL-triggered translocation of SREBP2 transcription factor to the nucleus, gene expression of several SREBP2 targets appeared to be simultaneously induced by HePC or edelfosine treatment. Remarkably, some pivotal genes involved in the cholesterogenic pathway were up-regulated by HePC and edelfosine, for example HMGCR, FDPS and FDFT1. Likewise, transcript levels of the SREBP2-responsive LDLR were also high after HePC and edelfosine treatment in a time-dependent manner. Incubation with HePC was previously reported to increase the level of expression of HMGCR in a time-dependent manner in the HepG2 cell line (Carrasco *et al.*, 2008). It is noticeable that exposure of these cells to any of the assayed APLs increased the expression of the SREBP2-responsive proteins HMGCR and LDLR after 24 h treatment, compared with the untreated cells (Figure 6). These results provide a mechanistic basis for the enhancement of cholesterol synthesis and LDL-cholesterol uptake after exposure of the cells to APLs.

Lipid biosynthesis may alter membrane lipid composition affecting lipid microdomain distribution or the amount of plasma membrane rafts. It has been previously reported that alteration in cholesterol biosynthesis affects lipid raft structure and function (Sánchez-Wandelmer *et al.*, 2009). Therefore, we extended our studies to analyse the possible effect of HePC exposure on the lipid composition of membrane raft and non-raft domains. In the present work, we have obtained raft and non-raft fractions from HepG2 cells by using a free-detergent method; these non-detergent lipid rafts were shown to be enriched in both cholesterol and sphingomyelin, relative to bulk plasma membrane. As above mentioned, HePC exerted an important cholesterogenic effect and we measured the effect of this action on cholesterol distribution between the different membrane domains. Our results revealed higher cholesterol content in raft fractions isolated from HePC-treated cells, compared with control cells, while sphingomyelin levels practically did not change. These effects are of considerable interest because cholesterol is assumed to be a critical stabilizing component in several types of lipid microdomains (Bakht *et al.*, 2007; Pike, 2009). For instance, depletion of cholesterol by methyl-β-cyclodextrin results in raft

disruption and subsequent malfunction of numerous signal transduction pathways (Gajate *et al*., 2009; Park *et al*., 2009). Interestingly, enrichment of the membrane with cholesterol also destabilizes membrane rafts (Matkó and Szöllösi, 2005).

As cholesterol and sphingomyelin content are critical for the integrity and functionality of membrane lipid rafts, the disturbance of the cholesterol/ sphingomyelin ratio could alter signalling pathways associated to these membrane domains. Although further studies must be carried out, the changes induced by APLs on lipid composition of membrane rafts, in part due to alterations in the cholesterol content of cells, as indicated in this work, can destabilize such rafts and undoubtedly modulate their properties, thereby disturbing cell function in tumour cells.

Acknowledgements

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Conflict of interest

None disclosed.

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4.3. Antitumoral alkylphospholipids downregulate genes associated with cholesterol export and catabolism in human hepatoma cells

In this work we deep on cholesterol metabolic pathways that could be affected by APLs such as the synthesis, catabolism, uptake and efflux. The experimental model was the hepatoma HepG2 cell line and we mainly performed this work by two different ways: 1) Analysis of the expression state in genes linked with cholesterol synthesis, catabolism and uptake and how APLs down/up regulate them. 2) Radiolabeling assays to measure cholesterol efflux to lipoprotein acceptor. For the gene expression studies, HepG2 cells were treated with HePC, edelfosine or ErPC for 6 and 24 h. Then, mRNA extraction and reverse-transcription to cDNA were preformed. This cDNA was applied to arrays from SABiosciences containing SYBR Green-optimized primer sets of genes related with apoptosis and cholesterol metabolism, for qRT-PCR. Several genes were found to be appreciably changed by APLs, when compared to control. For example, many cholesterol biosynthesis were induced: CYP51A1, DHCR7, DHCR24, FDFT1, FDPS, HMGCR, HMGCS1, IDI1, MVD, MVK, NSDHL, PRKAG2 and TM7SF2. On the other side, genes in charge of the catabolism and exporting of the cholesterol such as ABCA1, APOA1, APOA2, APOE , APOF, CETP, LCAT, AKR1D1, CYP7A1, CYP39A1, HMGCS2, INSIG2, LRPAP1, MBTPS1, NR0B2, NR2B1, SCAP, SORL1 and SREBP-1c were repressed. The second approach in this study was the measurement of the efflux of radiolabeled cholesterol through ABCA1 transporter to its specific acceptor ApoA1, with the final extracellular lipoprotein formation. Interestingly, there was a significant decrease in the efflux of cholesterol after 24 h APL-treatment in HepG2 cells.

As a summary, this work reports that treatment with antiproliferative lipid analogs after 6 to 24 hours induces cholesterol anabolism and uptake whereas catabolism and efflux are repressed. In other words, APL-treated HepG2 cells decrease cholesterol depleting processes, increasing at the same time its arrival and keeping. This together suggests that homeostatic response is extensively altered in the cells treated with these agents, probably in relation with the alterations described before regarding the cholesterol trafficking.

Antitumoral alkylphospholipids downregulate genes associated with cholesterol export and catabolism in human hepatoma cells.

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Abstract

Alkylphospholipid (APL) analogues are a new class of membrane-directed synthetic compounds with a variety of biological actions and clinical applications. In particular, these agents are promising candidates in cancer treatment. We have demonstrated that after prolonged treatment APLs alter intracellular cholesterol transport and metabolism in the HepG2 human hepatoma cell line, which leads to an accumulation of cholesterol inside the cell and thereby a disturbance of membrane lipid microdomains. Thus we have investigated the influence of different APLs upon the gene expression of key proteins involved in lipoprotein metabolism and cholesterol homeostasis in HepG2 cells. Quantitative real-time PCR analysis with a pathway-focused PCR array system was used to measure relative changes in the mRNA expression of a number of genes related to cholesterol metabolism and transport. We compared the gene expression profiles of HepG2 cells treated with miltefosine, edelfosine or perifosine for 6 h and 24 h with the profile of control cells. We found that APLs have pleiotropic effects on cholesterol transport and metabolism. The pathways for cholesterol biosynthesis and LDL-cholesterol uptake are both transcriptionally upregulated by the three APLs assayed. Another significant finding is that both the catabolism of cholesterol to bile acids and lipoprotein-associated cholesterol export are impaired by APLs, which may well contribute to the higher cell cholesterol levels induced by these compounds. In summary, APLs stimulated cholesterol biosynthesis and uptake at the same time as they downregulated the main pathways for excess cholesterol removal in HepG2 cells, ultimately leading to deregulation of cell cholesterol homeostasis.

Keywords: Alkylphospholipids; cholesterol homeostasis; PCR array system; HepG2 cells.

Introduction

A variety of mechanisms have been suggested to explain the activity of antitumoral alkylphospholipids (APLs), which can induce apoptosis and/or cell growth arrest (Barratt et al., 2009; van Blitterswijk and Verheij, 2008). Results obtained in our laboratory indicate that miltefosine (hexadecylphosphocholine) displays cytostatic activity and causes apoptosis in HepG2 cells (Jiménez-López et al., 2006, 2002). Likewise, treatment with miltefosine interferes with the synthesis of phosphatidylcholine via both CDP-choline and phosphatidylethanolamine methylation (Jiménez-López et al., 2004, 2002). With regard to sphingolipid metabolism, miltefosine hinders the formation of sphingomyelin, which promotes the intracellular accumulation of ceramide. We have demonstrated that treatment with miltefosine strongly impedes the esterification of cholesterol and that this effect is accompanied by a considerable increase in its synthesis, which leads to higher cholesterol levels in HepG2 and Vero cells (Jiménez-López et al., 2006). These alterations appear to be due to miltefosine's early impairment of cholesterol transport from the plasma membrane to the endoplasmic reticulum (ER), which causes a deregulation of cholesterol homeostasis (Carrasco et al., 2008; Marco et al., 2009). In a similar way to miltefosine, other synthetic APLs such as edelfosine, erucylphosphocholine and perifosine show growth inhibitory effects on HepG2 cells. All the APLs tested inhibit the arrival of plasma-membrane cholesterol at the ER, which induces a significant cholesterogenic response in HepG2 cells (Carrasco et al., 2010). Thus, membrane-targeted APLs share a common active mechanism which disrupts intracellular cholesterol homeostasis. The accumulation of cholesterol within the cell and a reduction in the biosynthesis of phosphatidylcholine and sphingomyelin alter the ratio of choline-bearing phospholipids to cholesterol, which is critical for the integrity and functionality of certain membrane microdomains such as lipid rafts. Therefore alterations induced by APLs in lipid homeostasis, leading probably to a disturbance in the native membrane structure, may well affect signalling processes vital to cell survival and growth (Jiménez-López et al., 2010; Ríos-Marco et al., 2011).

Recent progress in studying the transcriptional regulation of lipid catabolism has revealed the crucial role of non-steroidal nuclear receptors such as peroxisome

proliferator-activated receptors (PPAR/NR1C) and liver X receptors (LXR/NR1H), whereas members of the sterol regulatory element-binding protein (SREBP) family have been established as key regulators of lipid synthesis. The aim of the present study was to investigate whether, in addition to the processes leading to an increase in cholesterol levels (synthesis and uptake), the pathways leading to the removal of cholesterol from the cells (catabolism and export) are likewise altered after incubation with three different APLs. Quantitative real-time PCR was used to determine the relative expression levels of a number of genes related to cholesterol metabolism and its transport in and out of the cell. We found significant changes in several sterol-sensitive transcription factors and other proteins involved in the regulation of cell cholesterol homeostasis.

Material and Methods

Chemicals and reagents

Foetal bovine serum (FBS) was from The Cell Culture Company (Pasching, Austria); minimal essential medium (MEM) and apolipoprotein (apo)-A1 from human plasma were from Sigma-Aldrich (Madrid, Spain); [7(n)^{3}-^{3}H]cholesterol from Perkin Elmer (Massachusetts, USA); miltefosine from Cayman Chemical (Ann Arbor, MI, USA); edelfosine from Calbiochem (Nottingham, UK) and perifosine from Selleck Chemicals (London, ON, Canada).

Cell culture

The human hepatoma HepG2 cell line was from The European Collection of Animal Cell Cultures (Salisbury, UK). Cells were cultured in MEM containing 10% heat-inactivated FBS supplemented with 2 mM L-glutamine, 1% non-essential amino acids, 100 U/ml penicillin and 100 µg/ml streptomycin. They were grown in a humidified atmosphere with 5% CO_2 at 37ºC and subcultured at a ratio of 1:10 once a week. Cells were plated on tissue-culture dishes (Nunc^{TM}, from LabClinics SA, Barcelona, Spain) at a density of 5 x 10^4 cells/cm^2 and kept in culture medium before use in experimental assays at approximately 70% confluence.

Quantification of mRNA using a real-time PCR Array System

Exponentially growing HepG2 cells were incubated with MEM containing 10% FBS in the absence [phosphate-buffered saline (PBS) as vehicle] or presence of 25 μM miltefosine, edelfosine or perifosine for 6 h and 24 h; triplicate samples were prepared for each drug treatment for each time. Total RNA was isolated using the RNeasy Mini kit (Qiagen) and reverse-transcribed into cDNA using the RT^2 First Strand kit from SABiosciences (Frederick, MD, USA), which includes both oligo-dT and random hexamer primers. SYBR Green real-time PCR was conducted in a MyiQ^{TM}2 Real-Time PCR Detector (Bio-Rad) using the Human Lipoprotein Signalling & Cholesterol Metabolism RT^2 Profiler^{TM} PCR Array from SABiosciences, which profiles the expression of a focused panel of genes related to lipoprotein transport and cholesterol metabolism. The array also includes sets of replicate control wells that specifically detect non-transcribed genomic DNA contamination, the efficiency of reverse transcription and the efficiency of positive PCR reactions, and specific primer sets for the amplification of five housekeeping gene transcripts: β-2-microglobulin, hypoxanthine phosphoribosyltransferase 1, ribosomal protein L13a, glyceraldehyde-3-phosphate dehydrogenase and β-actin. We also analysed some other genes of interest that were not represented on the PCR array. These included the genes encoding the alpha isoforms of LXR, PPAR and retinoid X receptor (RXR/NR2B), the regulation of which was examined using the RT^2 qPCR Primer Assays from SABiosciences and glyceraldehyde-3-phosphate dehydrogenase as reference. As a quality control, a dissociation (melting) curve was acquired immediately after the PCR cycling program. The relative expression ratio was determined from the averaged threshold cycle values, i.e. $\Delta\Delta C_T$ based fold-change calculations, using an integrated web-based software package from SABiosciences for the PCR array data analysis.

Measurement of cholesterol efflux to apoA1

HepG2 cells at around 60% confluence were labeled with 1 μCi/ml [^{3}H]cholesterol in serum-free medium containing 0.2% fatty acid-free bovine serum albumin (BSA) for 24 h. The medium was removed and the cells were washed three times with PBS, and twice with PBS containing 0.1% fatty acid-free BSA. The radiolabeled cholesterol was

equilibrated among cellular pools by incubating the cells with serum-free medium supplemented with 0.2% fatty acid-free BSA for 16 h. Cells were then incubated with MEM containing 10% FBS in the absence (PBS as vehicle) or presence of APLs for 24 h. Subsequently, cells were extensively washed with 0.1% fatty acid-free BSA in PBS and then incubated with serum-free medium containing 10 μg/ml lipid-free apoA1 as cholesterol acceptor. ABCA1 (ATP-binding cassette transporter isoform A1)-mediated cholesterol efflux was determined by measuring the release of radioactive cholesterol into the medium at various experiment intervals (0, 0.5, 1, 2 and 4 h). Aliquots of the incubation medium were taken off at times indicated and centrifuged at 12000 rpm for 10 min to remove cell debris, and the amount of labeled cholesterol recovered in the medium was measured with a Beckman 6000-TA liquid scintillation counter; the time zero radiactivity in the medium was subtracted at the different time points. Afterwards lipids were extracted from the cells following the procedure of Bligh and Dyer (1959), and the radioactivity counted by liquid scintillation. Results were expressed in terms of the percentage of labelled total cholesterol transferred from cells to the medium as a function of time.

Results

Expression of genes related to the synthesis and receptor-mediated uptake of cholesterol in HepG2 cells

In a previous paper we reported that both miltefosine and edelfosine trigger a significant cholesterogenic response in HepG2 cells through the activation of the SREBP2 transcription factor (Carrasco et al., 2010). Moreover, cell-surface low-density lipoprotein receptor (LDLR) activity increased after exposure to miltefosine, as measured by the uptake of [^{3}H]cholesteryl linoleate-LDL (Carrasco et al., 2008). We have now undertaken a comparative study using different APLs to identify a variety of cholesterol-related genes the transcripts of which increase or decrease in APL-treated HepG2 cells. Table 1 shows the ability of membrane-targeted APLs to stimulate mRNA expression of key proteins involved in cholesterol biosynthesis and LDL-cholesterol uptake. Thus, several genes listed in Table 1 encode enzymes such as cytosolic 3-

hydroxy-3-methylglutaryl (HMG)-CoA synthase (HMGCS1), HMG-CoA reductase (HMGCR), mevalonate kinase (MVK), diphosphomevalonate decarboxylase (MVD), isopentenyl-diphosphate isomerase (IDI1), farnesyl diphosphate synthase (FDPS), squalene synthase (farnesyl-diphosphate farnesyltransferase, FDFT1) and 7-dehydrocholesterol reductase (DHCR7), which are transcriptionally-regulated, rate-determining enzymes in the cholesterol biosynthetic pathway (Horton et al., 2003; Sato and Takano, 1995). As far as cholesterol uptake is concerned, higher gene expression levels of LDLR and the Niemann-Pick C1-like 1 (NPC1L1) transmembrane protein, which are involved in cholesterol transport and absorption, were observed after 6 and 24 hours' treatment with miltefosine, edelfosine or perifosine compared to transcription levels in untreated cells. Furthermore, mRNA expression of some ligand apoproteins for high-density lipoprotein (HDL) or low-density lipoprotein (LDL) receptors such as apoA4 and apoB appeared to increase after exposure to APL. Apart from that, after APL treatment we found an increase in the gene expression of the secretory proprotein convertase subtilisin/kexin 9 (PCSK9), which binds to LDLRs and promotes their degradation (Mousavi et al., 2009), and likewise for SCARF1, a F-type scavenger receptor that binds and internalizes modified forms of LDL (Plüddemann et al., 2007).

Transcriptional modulation of SREBP2 and the PPAR members α and δ (also known as β) by APLs may be particularly important in explaining the cholesterol-increasing effects that result from APL exposure (Table 1). The ubiquitously expressed SREBP2 transcription factor controls cholesterol homeostasis in mammalian cells by stimulating the transcription of genes for cholesterol synthesis and the LDLRs (Horton et al., 2003), whilst PPARs are lipid sensors that regulate lipoprotein, fatty-acid and carbohydrate metabolism (Li and Chiang, 2009). PPARα is highly expressed in the liver and plays a key role in fatty-acid catabolism by upregulating the expression of genes involved in mitochondrial fatty-acid oxidation (Hashimoto et al., 1999). PPARδ activity is associated generally with the transcriptional repression of basal and ligand-induced PPARα and PPARγ and thus it modulates nuclear receptor signalling and global lipid homeostasis (Shi et al., 2002).

Expression of genes related to the catabolism of cholesterol into bile acids and lipoprotein-mediated cholesterol efflux in HepG2 cells

Our next step was to examine the possible effects on cholesterol catabolism and export mediated by lipoproteins after 6 and 24 hours' incubation of hepatoma cells in the presence of three different APLs. We found that they repressed mRNA expression of several proteins involved in the removal of excess cholesterol via HDL particles (Table 2). Thus, APL treatment appears to inhibit lipoprotein-associated cholesterol efflux, since transcripts for (i) the cholesterol-efflux pump ABCA1, (ii) HDL-linked apolipoproteins such as apoA1, apoA2, apoE and apoF, and (iii) HDL-bound enzymes such as cholesteryl ester transfer protein (CETP) and lecithin-cholesterol acyltransferase (LCAT) decreased after exposure to the APLs in question. Cholesterol efflux from hepatic cells is receiving increasing attention since evidence is emerging that a large pool of cholesterol released from cells to nascent HDL particles comes from the liver (Lewis and Rader, 2005). ABCA1 functions as a transporter to export excess cholesterol. Indeed, lipidation of apoA1 by ABCA1 is a rate-limiting step for plasma HDL assembly in reverse cholesterol transport from peripheral tissues as well as in cholesterol efflux from liver cells (Cavelier et al., 2006). To verify the data emerging from these recent gene expresion experiments we studied the influence of APLs on ABCA1-mediated cholesterol efflux activity. Treatment with different APLs for 24 h was found to decrease to some extent the native ABCA1-dependent cholesterol efflux in HepG2 cells (Fig. 1).

A lower rate of cholesterol catabolism for biliary excretion may also be involved in the APL-induced accumulation of cholesterol inside HepG2 cells. As can be seen from Table 2, treatment with APLs greatly repressed the expression of transcripts for the aldo-keto 5β-reductase AKR1D1, the regulatory enzyme cholesterol 7α-hydroxylase (CYP7A1) and the oxysterol 7α-hydroxylases CYP7B1 and CYP39A1, which all play a role in synthesizing primary bile acids from cholesterol. Treatment with APLs significantly repressed the expression of other genes encoding proteins that are also involved in the regulation of cholesterol metabolism (Table 2): (i) lower LXRα expression was observed after 24 hours' incubation with either miltefosine or edelfosine, which was sometimes accompanied by a decrease in the expression of other

members of the non-steroidal nuclear receptor family, such as the small heterodimer partner (SHP) or RXRα, which interact with LXRα to form heterodimers that regulate the expression of target genes containing LXR response elements, to control cell lipid homeostasis (Oosterveer et al., 2010); (ii) transcript levels for HMGCS2, a nuclear-encoded mitochondrial enzyme that catalyzes the first reaction of ketogenesis, were also diminished after APL treatment, which may increase the supply of acetyl-CoA to the cytosol for fatty-acid and cholesterol biosyntheses; (iii) mRNA expression of INSIG2 (encoding an ER anchor protein very similar to Insig1) was also lowered by APLs, which would facilitate the ER-to-Golgi transport of the SREBP-Scap (SREBP cleavage-activating protein) complex and thus the proteolytic processing of SREBP to its active form for translocation to the nucleus; (iv) gene expression of the sterol-regulated proteins Scap and MBTPS1 (membrane-bound transcription factor peptidase, site 1; S1P/PCSK8) also decreased after incubation of the HepG2 cells with APLs, these proteins being involved in the activation of SREBP when cholesterol levels fall; (v) finally, two other lipoprotein receptor-associated proteins, LRPAP1 (LDLR-related protein associated protein 1) and SORL1 (sortilin-related receptor containing LDLR class A repeats) underwent decreases in gene expression in APL-treated cells (Table 2).

Discussion

Cholesterol levels ultimately result from a balance between uptake, efflux and endogenous metabolism. We have previously demonstrated that the incubation of HepG2 cells with different APLs leads to an intracellular accumulation of cholesterol mainly due to the inhibition of cholesterol transport from the plasma membrane to the ER, where cholesterol-sensing enzymes are located; as a result the receptor-dependent uptake and *de novo* synthesis of cholesterol are increased, leading to the disruption of cell cholesterol homeostasis (Carrasco et al., 2010). Stimulation of cholesterogenesis induced by prolonged exposure of HepG2 cells to APLs is also accompanied by the transcriptional repression of cholesterol catabolism and export pathways, as shown in this study (Tables 1 and 2). Several homeostatic systems appear to be either directly or indirectly regulated by excess (active) plasma-membrane cholesterol, including the

regulation of transcription for cell cholesterol homeostasis (Lange et al., 2009). Within this context, membrane-intercalating amphipathic lipids bearing saturated alkyl chains and large polar head groups, such as lysophospholipids and APLs, inhibit the inward transfer of membrane cholesterol and homeostatic responses (Carrasco et al., 2010; Steck and Lange, 2010).

The PCR array approach has allowed us to define a set of cholesterol-related genes, the transcription of which changes in HepG2 cells after 6 or 24 hours' incubation in the presence of three different APLs, thereby revealing some activities that are likely to be modulated by APLs to maintain intracellular cholesterol homeostasis. The pathways for cholesterol biosynthesis and LDL-cholesterol uptake were both transcriptionally upregulated by APLs (Table 1). Transcription factors called SREBPs are ER membrane proteins which, following activation, are known to enhance directly the co-ordinated transcription of genes needed for LDL-cholesterol endocytosis mediated by LDLRs and the synthesis of cholesterol and fatty acids (Horton et al., 2003). The predominant forms in the liver are SREBP2 and SREBP-1c, which preferentially regulate genes involved in cholesterol and fatty-acid biosynthesis respectively (Horton et al., 2002). The activity of each membrane-bound SREBP isoform is regulated by sterols and Scap, a SREBP chaperone; in fact, Scap serves both as a sensor for free cholesterol in the ER and as an escort for SREBP. In the presence of cholesterol, SREBPs are sequestered in the ER. Upon the absence of any sterol signal in the ER, on the other hand, Scap does not bind to the anchor protein Insig (insulin induced gen) but escorts SREBP from the ER to the Golgi, where two sequential proteolytic cleavage events, mediated by site-1 (S1P) and site-2 (S2P) proteases, cause the activation of SREBP and its subsequent transport into the nucleus, where it activates transcription by binding to sterol regulatory element (SRE) sequences in the promoter/enhancer region of multiple target genes (Horton et al., 2002).

We have reported elsewhere that the ER membrane becomes depleted of cholesterol after APL treatment because plasma membrane-to-ER transport is impaired in the presence of APLs (Carrasco et al., 2010), and that this interference leads to the activation of cholesterol biosynthesis mediated directly by SREBP2, the transcripts of which increased after the incubation of cells with APLs. In fact, many of the mRNAs

encoding enzymes in the cholesterogenic pathway were upregulated in the presence of different APLs (Table 1). At the same time, the SRE-bearing LDLR and PCSK9 genes were transcriptionally upregulated by mature SREBP2 in the presence of APLs (Mousavi et al., 2009; Sato and Takano, 1995). PCSK9 is a recently discovered protein involved in cholesterol homeostasis (Mousavi et al., 2009). The best understood function of this secreted protein is the downregulation of hepatic LDLRs and possibly other surface receptors; PCSK9 prevents the recycling of LDLRs from endosomes, leading to lysosomal degradation of hepatic LDLRs and thence to increased plasma LDL-cholesterol levels. Like many other sterol-responsive genes, the promoter region of PCSK9 contains a SRE that binds SREBP2. Therefore, higher SREBP2 activity might account for the increase in the mRNA expression of PCSK9 in HepG2 cells as an additional control on cholesterol homeostasis after exposure to APLs (Table 1). Treatment with APLs also downregulates LRPAP1 (Table 2), a subunit of LDLR-related protein (LRP) that is also involved in cholesterol clearance; LRPAP is to be found predominantly in the ER and might function as a chaperone protein to nascent LRP during its intracellular transport, playing an inhibitory role over LRP activity (Pandey et al., 2008).

In our present study, the incubation of hepatoma cells with different APLs stimulated transcriptionally the expression of NPC1L1, which may contribute to the higher internalization of cholesterol in the APL-treated cells, in a similar way to that in which LDLR does. NPC1L1 is an apically-located sterol transporter essential for intestinal cholesterol absorption, which is also highly expressed in the human liver. This transporter protein facilitates the transfer of extracellular cholesterol to the cytoplasm of intestinal and liver cells, the NPC1L1-dependent sterol uptake being regulated by the cell cholesterol content (Betters and Yu, 2010). In fact, NPC1L1 appears to function as a free-cholesterol receptor in the plasma membrane, resembling Scap, the sterol sensor in the ER.

The conversion of HMG-CoA to mevalonate, catalyzed by HMGCR, is an early rate-limiting step in the cholesterol biosynthetic pathway. This enzyme contains a sterol-sensing domain, as does Scap, and is also negatively regulated by binding to Insig, which accelerates its ubiquitination and proteasomal degradation (DeBose-Boyd,

2008). Thus, Insigs are ER-resident proteins that negatively regulate Scap and HMGCR; humans express two forms, Insig1 and Insig2, which are essential mediators of cholesterol feedback regulation, controlling both the activation of SREBP through ER retention and sterol-accelerated degradation of HMGCR (DeBose-Boyd, 2008; Yang et al., 2002). Accordingly, the lower expression of the ER-retention oxysterol-binding protein Insig2 in the presence of APLs would facilitate the movement of the SREBP-Scap complex (recruited into ER-derived transport vesicles) to the Golgi and would retard the degradation of endogenous HMGCR (DeBose-Boyd, 2008). As indicated above, SREBPs function as master regulators of cholesterol and fatty-acid synthesis. SREBP2 upregulates the expression of most cholesterol biosynthetic enzymes as well as the LDLR, whereas SREBP-1c stimulates the transcription of genes required for fatty-acid synthesis. The central role that SREBPs play in the control of lipid synthesis is highlighted by the multiple inputs to SREBP activity from other signalling pathways. The nuclear hormone receptors LXR and RXR function as a heterodimer to upregulate SREBP-1c in response to cholesterol overloading, possibly to increase the supply of unsaturated fatty acids (mainly oleate) needed to esterify cholesterol for storage and packaging into lipoproteins for export (Horton et al., 2002; Repa et al., 2000).

Cells express cytochrome P450-dependent cholesterol oxidases that convert cholesterol into more hydrophilic bile acids (in liver cells) or oxysterols, which are then secreted. Bile-acid synthesis in the liver is the major pathway for cholesterol output. The biotransformation of cholesterol to primary bile acids occurs mainly via two pathways by which cholesterol is converted into cholic acid: the classic pathway, initiated by microsomal CYP7A1 (rate-limiting step); and an alternative pathway, initiated by mitochondrial sterol 27-hydroxylase (CYP27), the product of which, 27-hydroxycholesterol, is then 7α-hydroxylated by microsomal oxysterol 7α-hydroxylase CYP7B1 (Pandak et al., 2002). Another microsomal oxysterol 7α-hydroxylase is CYP39A1, which preferentially catalyzes the 7α-hydroxylation of 24-hydroxycholesterol and has been put forward as playing a role in the alternative bile-acid synthetic pathway in the liver (Li-Hawkins et al., 2000). In our PCR analysis we found that the three microsomal sterol 7α-hydroxylases assayed, CYP7A1, CYP7B1

and CYP39A1, were all transcriptionally downregulated in HepG2 cells after exposure to APLs. Thus, any misregulation of bile-acid synthesis might further contribute to the accumulation of cholesterol in these cells.

Mitochondria convert cholesterol into steroids, bile acids and oxysterols and, like the ER, are principal targets for intracellular homeostatic signalling by active (escape tendency) cholesterol; overall, active plasma-membrane cholesterol must be conveyed to the ER and mitochondrial membranes to elicit the regulatory responses (Steck and Lange, 2010). Side-chain oxidized oxysterols such as 27-hydroxycholesterol are more water-soluble than cholesterol and contribute to the retro-inhibition of cholesterol synthesis to prevent cholesterol accumulation (Lange et al., 2009). As indicated above, this oxysterol is synthesized by mitochondrial CYP27, the rate-determining enzyme that initiates the alternative bile-acid synthetic pathway (Xu et al., 1999). Insig-oxysterol complexes associate with Scap-cholesterol in the ER to prevent SREBP transport, whilst, on the other hand, 27-hydroxycholesterol production decreases in response to lower active plasma-membrane cholesterol and thus leads to the release of SREBP. Furthermore, side-chain oxysterol derivatives of cholesterol, synthesized in proportion to active cholesterol levels, may also regulate the abundance of cell sterols via the modulation of LXRα and LXRβ nuclear receptors (Edwards et al., 2002). In fact, the most likely physiological ligand for LXR in cholesterol-loaded cells is 27-hydroxycholesterol (Fu et al., 2001). The results obtained in our present work allow us to hypothesize that, apart from their capacity to impair plasma membrane-to-ER cholesterol traffic, APLs could also hinder cholesterol transport into mitochondria, thereby reducing the generation of 27-hydroxycholesterol and consequently oxysterol-dependent LXR activity. This effect would partially explain the lower gene expression of several LXR targets after exposure to APL; thus an analysis for transcript expression (Table 2) reveals downregulation by APLs of a variety of LXR targets that play a critical role in the maintenance of cholesterol homeostasis (Edwards et al., 2002; Hu et al., 2010), including ABCA1, apoA1, apoE, CETP and SREBP-1c.

Cholesterol efflux from cells onto various extracellular acceptors takes place by both passive and active mechanisms. Passive efflux occurs via uncatalyzed and facilitated pathways such as export mediated by the scavenger receptor SR-BI in the

plasma membrane. Cholesterol is also pumped from the cell to diverse acceptors by several members of the ABC transporter superfamily of plasma-membrane proteins, which involves an active and specific lipid efflux process. As has been reported by other authors, endogenous oxysterols are the physiological ligands that allow LXRs to sense any excess cell cholesterol, which then promote the expression of certain ABC transporters capable of expelling cholesterol from the cells. ABCA1 plays a key role in mediating the efflux of cholesterol and phospholipids to lipid-free apoA1, thereby initiating the assembly of nascent HDL particles (Cavelier et al., 2006; Vedhachalam et al., 2007). ApoE and certain lipoproteins may also serve as acceptor particles for ABCA1-dependent cholesterol efflux. Data in our present study demonstrate that exposure of HepG2 cells to APLs downregulates ABCA1 after 6 hours' treatment; likewise, ABCA1-mediated cholesterol efflux was shown to be reduced by APLs. These results strongly suggest that the efficiency of plasma-membrane cholesterol efflux by ABCA1, and hence lipoprotein-associated cholesterol export, are inhibited by APLs (Fig. 2). The reverse cholesterol transport pathway involves an initial uptake of cell cholesterol by HDL, where it is esterified before being transferred by CETP to LDL and very-low-density lipoproteins. The cholesteryl esters in the apoB-bearing lipoprotein pool are subsequently delivered to the liver and ultimately eliminated from the body as a component of bile. Most of the cholesteryl esters in plasma originate in HDL in the reaction catalyzed by LCAT. Interestingly, we also found in this study that when hepatoma cells were incubated with the different APLs assayed the result was a lower gene expression of both HDL-bound plasma enzymes, CETP and LCAT (Table 2).

Few publications have presented results deriving from gene expression studies after treatment with APLs although recently a genome-wide study investigated the impact of edelfosine and two glycosidated APLs on gene expression in HaCaT cells and identified multiple transcripts differently expressed after incubation with each APL for 24 h. These genes are mainly associated with lipid metabolism, cell development and differentiation, ion homeostasis and, as described for the first time, immune and inflammatory responses (Semini et al., 2011). The expression of many of the proteins involved in cholesterol transport and metabolism is controlled by SREBP and the LXR and PPAR nuclear receptors. To our knowledge, the effects of APLs upon the

expression of different LXR and PPAR members have not been previously described, neither is it known whether they might play a role in disrupting lipid homeostasis in the presence of APLs. RXR is an obligate and common partner of LXR and PPARα, acting in opposite manner with regard to fatty-acid metabolism: SREBP-1c activated by LXR regulates fatty-acid synthesis, whereas PPARα controls fatty-acid degradation; moreover, cross-talk between both nuclear receptors permits an efficient reciprocal regulation of lipid degradation and lipogenesis (Ide et al., 2003; Yoshikawa et al. 2003). As far as this is concerned, we found that gene expression of the PPARα isoform increased whilst the expression of both LXRα and RXR diminished after 6 hours' incubation of the hepatoma cells in the presence of miltefosine.

In summary, APLs stimulated cholesterol biosynthesis and uptake at the same time as they inhibited the main pathways for cholesterol export and bile-acid synthesis in hepatoma HepG2 cells. Therefore, they appear to act by downregulating several cholesterol-removal pathways in HepG2 cells (Fig. 2). The full identification and further characterization of these changes may give important clues to the sequence of events that result from APL interference leading to this deregulation of cholesterol homeostasis.

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Conflict of Interest Statement

None declared.

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	Relative expression (6 and 24 hours' treatment)					
Gene	**Miltefosine**		**Edelfosine**		**Perifosine**	
Cholesterol Biosynthesis	*6 h*	*24 h*	*6 h*	*24 h*	*6 h*	*24 h*
CYP51A1	1.49 ± 0.01[a]	1.41 ± 0.19[a]	~	1.63 ± 0.14[a]	1.17 ± 0.15	~
DHCR7	1.66 ± 0.04[a]	1.42 ± 0.16[a]	1.70 ± 0.08[a]	1.33 ± 0.05[a]	1.40 ± 0.22	~
DHCR24	1.63 ± 0.04	1.15 ± 0.19	1.11 ± 0.06	~	1.36 ± 0.05[a]	~
FDFT1	1.57 ± 0.21[a]	1.47 ± 0.08[a]	1.57 ± 0.21	1.47 ± 0.08	1.26 ± 0.17	~
FDPS	1.31 ± 0.09[a]	2.17 ± 0.08[a]	1.31 ± 0.09	2.17 ± 0.08[a]	1.26 ± 0.18	1.34 ± 0.27
HMGCR	1.88 ± 0.01[a]	1.73 ± 0.04[a]	2.15 ± 0.18[a]	1.76 ± 0.08[a]	1.36 ± 0.17	1.21 ± 0.17
HMGCS1	1.36 ± 0.43	2.07 ± 0.24[a]	1.08 ± 0.12	~	1.81 ± 0.34	2.15 ± 1.12
IDI1	2.23 ± 0.01[a]	1.81 ± 0.14[a]	1.40 ± 0.20	1.19 ± 0.16	1.44 ± 0.04[a]	~
MVD	1.87 ± 0.16[a]	1.60 ± 0.23[a]	2.28 ± 0.24[a]	1.93 ± 0.01[a]	1.66 ± 0.14[a]	1.28 ± 0.14
MVK	1.57 ± 0.18	1.52 ± 0.31	1.34 ± 0.14	1.30 ± 0.04[a]	1.46 ± 0.26	1.23 ± 0.10
NSDHL	1.84 ± 0.02[a]	1.46 ± 0.12[a]	1.79 ± 0.19[a]	1.31 ± 0.06	1.44 ± 0.21	1.13 ± 0.08
PRKAG2	1.17 ± 0.09	1.87 ± 0.31[a]	~	1.29 ± 0.03[a]	~	1.36 ± 0.10[a]
TM7SF2	1.67 ± 0.12[a]	1.22 ± 0.11	1.83 ± 0.04[a]	1.55 ± 0.10[a]	1.27 ± 0.15	1.45 ± 0.02
Cholesterol Transport						
APOA4	1.61 ± 0.01	~	~	1.35 ± 0.11	1.50 ± 0.08[a]	1.45 ± 0.11[a]
APOB	1.54 ± 0.23	1.27 ± 0.33	~	~	~	~
LDLR	1.32 ± 0.15	1.60 ± 0.13[a]	~	1.43 ± 0.13	1.28 ± 0.21	1.23 ± 0.13
NPC1L1	~	1.32 ± 0.28	~	1.37 ± 0.05[a]	1.33 ± 0.23	~
PCSK9	1.37 ± 0.12	1.36 ± 0.32	2.07 ± 0.10[a]	2.28 ± 0.17[a]	1.93 ± 0.19[a]	1.39 ± 0.15[a]
SCARF1	1.50 ± 0.09	1.29 ± 0.36	~	1.16 ± 0.01	~	1.41 ± 0.38
Cholesterol Metabolism						
PPARα	1.47 ± 0.12	~	n.d.	n.d.	n.d.	n.d.
PPARδ	1.42 ± 0.03[a]	1.23 ± 0.11	1.53 ± 0.09[a]	1.19 ± 0.05	1.38 ± 0.19	1.15 ± 0.03
SREBP2	~	1.34 ± 0.10[a]	1.31 ± 0.02	1.37 ± 0.08	1.10 ± 0.13	1.08 ± 0.17

Table 1. Genes whose transcripts increase in alkylphospholipid-treated HepG2 cells. Total RNA was prepared for each condition and reverse transcribed into cDNA as described in Material and Methods. The relative mRNA expression of each gene in alkylphospholipid-treated cells compared to the appropriate control is shown, indicating whether the relative change in mRNA expression occurred after 6 and/or 24 hours' incubation with 25 μM of each compound. Absence of effect is depicted by the symbol ~. Genes are grouped according to function and are listed in alphabetical order under each heading. [a]$P < 0.05$ compared to control.

CYP51A1 (cytochrome P450, family 51, subfamily A, polypeptide 1); DHCR7 (7-dehydrocholesterol reductase); DHCR24 (24-dehydrocholesterol reductase); FDFT1 [farnesyl-diphosphate farnesyltransferase 1 (squalene synthase)]; FDPS [farnesyl diphosphate synthase (farnesyl pyrophosphate synthetase, dimethylallyltranstransferase, geranyltranstransferase)]; HMGCR (3-hydroxy-3-methylglutaryl-coenzyme A reductase); HMGCS1 [3-hydroxy-3-methylglutaryl-coenzyme A synthase 1 (soluble)]; IDI1 (isopentenyl-diphosphate delta isomerase 1); MVD (mevalonate (diphospho) decarboxylase); MVK (mevalonate kinase); NSDHL (NAD(P) dependent steroid dehydrogenase-like); PRKAG2 (protein kinase, AMP-activated, gamma 2 non-catalytic subunit); TM7SF2 (transmembrane 7 superfamily member 2).

APOA4 (apolipoprotein A-IV); APOB (apolipoprotein B); LDLR (low-density lipoprotein receptor); NPC1L1 [NPC1 (Niemann-Pick disease, type C1, gene)-like 1]; PCSK9 (proprotein convertase subtilisin/kexin type 9); SCARF1 (scavenger receptor class F, member 1).

PPARα (peroxisome proliferator-activated receptor alpha); PPARδ (peroxisome proliferator-activated receptor delta); SREBP2 (sterol regulatory element-binding protein 2).

	Relative expression (6 and 24 hours' treatment)					
Gene	**Miltefosine**		**Edelfosine**		**Perifosine**	
Reverse Cholesterol Transport	*6 h*	*24 h*	*6 h*	*24 h*	*6 h*	*24 h*
ABCA1	0.78 ± 0.07	0.79 ± 0.06	0.54 ± 0.09	~	0.52 ± 0.07[a]	0.75 ± 0.09
APOA1	~	0.86 ± 0.16	0.84 ± 0.05	0.77 ± 0.06	0.80 ± 0.11	0.70 ± 0.03
APOA2	~	0.88 ± 0.09	0.76 ± 0.07	0.80 ± 0.01	0.81 ± 0.12	0.71 ± 0.04
APOE	0.77 ± 0.02	0.86 ± 0.25	~	~	~	~
APOF	0.89 ± 0.11	0.84 ± 0.20	0.71 ± 0.07	0.65 ± 0.04[a]	0.65 ± 0.13	0.63 ± 0.09
CETP	0.82 ± 0.07	0.84 ± 0.18	~	0.86 ± 0.04	0.68 ± 0.12	0.66 ± 0.05[a]
LCAT	~	0.87 ± 0.25	~	0.66 ± 0.04	0.84 ± 0.08	0.77 ± 0.05
Cholesterol Catabolism						
AKR1D1	~	0.72 ± 0.21	0.59 ± 0.04	0.31 ± 0.02[a]	0.71 ± 0.09	0.39 ± 0.01[a]
CYP7A1	~	0.62 ± 0.22	0.55 ± 0.01[a]	0.46 ± 0.02[a]	0.62 ± 0.06	0.31 ± 0.04[a]
CYP7B1	~	0.52 ± 0.31	~	0.85 ± 0.04	n.d.	n.d.
CYP39A1	~	~	~	0.81 ± 0.03	~	0.91 ± 0.05
Cholesterol Metabolism						
HMGS2	~	0.63 ± 0.34	~	0.62 ± 0.05	~	0.64 ± 0.18
INSIG2	~	~	~	~	0.79 ± 0.09	0.87 ± 0.03
LRPAP1	~	0.80 ± 0.09	0.55 ± 0.08[a]	0.58 ± 0.07[a]	0.88 ± 0.14	0.81 ± 0.01[a]
MBTPS1 (S1P/PCSK8)	~	0.83 ± 0.07	~	~	~	~
NR0B2 (SHP)	0.82 ± 0.03[a]	0.83 ± 0.17	0.63 ± 0.05	0.81 ± 0.02	0.72 ± 0.12	0.71 ± 0.06
NR1H3 (LXRα)	~	0.62 ± 0.06	0.61 ± 0.09	0.63 ± 0.11	~	~
NR2B1 (RXRα)	~	0.69 ± 0.02	~	~	~	~
SCAP	0.92 ± 0.01	0.85 ± 0.13	0.58 ± 0.09	0.76 ± 0.02	~	0.85 ± 0.01
SORL1	~	~	0.61 ± 0.06	0.76 ± 0.07	0.86 ± 0.11	0.70 ± 0.15
SREBP-1c	0.78 ± 0.04[a]	0.84 ± 0.14	~	~	0.90 ± 0.18	~

Table 2. Genes whose transcripts decrease in alkylphospholipid-treated HepG2 cells See legend to Table 1.

ABCA1 (ATP-binding cassette, sub-family A (ABC1), member 1); APOA1 (apolipoprotein A-I); APOA2 (apolipoprotein A-II); APOE (apolipoprotein E); APOF (apolipoprotein F); CETP (cholesteryl ester transfer protein, plasma); LCAT (lecithin-cholesterol acyltransferase).

AKR1D1 [aldo-keto reductase family 1, member D1 (delta 4-3-ketosteroid-5-beta-reductase)]; CYP7A1 (cytochrome P450, family 7, subfamily A, polypeptide 1); CYP39A1 (cytochrome P450, family 39, subfamily A, polypeptide 1).

HMGCS2 [3-hydroxy-3-methylglutaryl-coenzyme A synthase 2 (mitochondrial)]; INSIG2 (insulin-induced gene 2); LRPAP1 (low-density lipoprotein receptor-related protein associated protein 1); MBTPS1 (membrane-bound transcription factor peptidase, site 1; S1P/PCSK8); NR0B2 (nuclear receptor subfamily 0, group B, member 2; SHP); NR1H3 (nuclear receptor subfamily 1, group H, member 3; LXRα); NR2B1 (retinoid X receptor alpha; RXRα); SCAP (SREBP cleavage-activating protein); SORL1 (sortilin-related receptor containing LDLR class A repeats); SREBP-1c (sterol regulatory element-binding protein 1c).

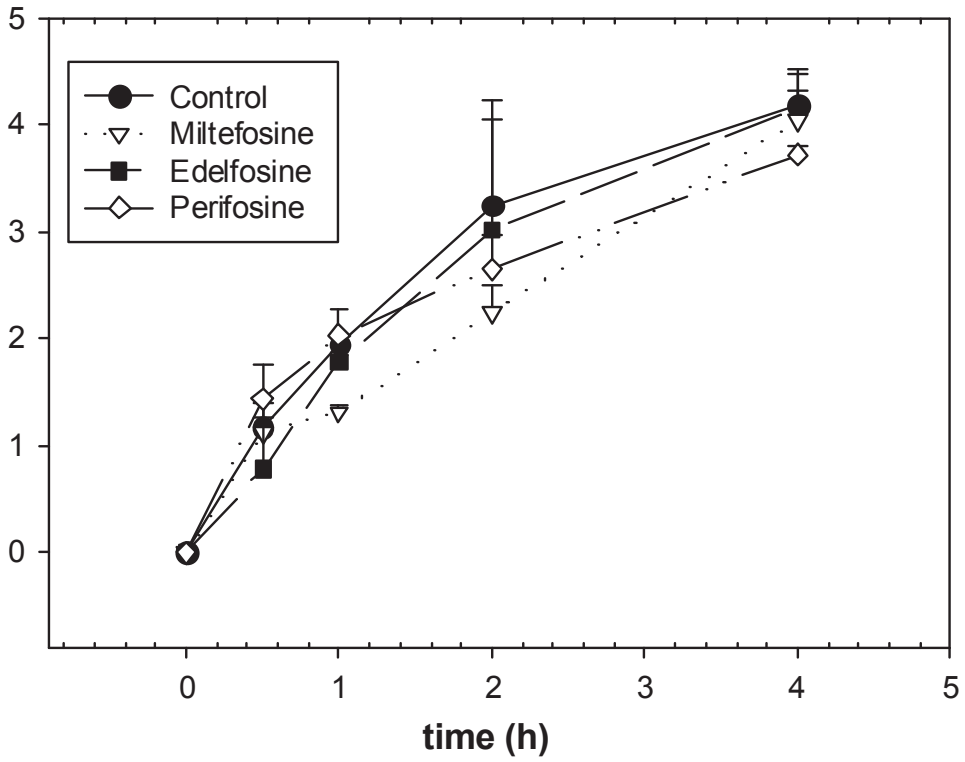

Figure 1. Treatment with alkylphospholipids decreases ABCA1-mediated cholesterol efflux activity in hepatoma HepG2 cells.

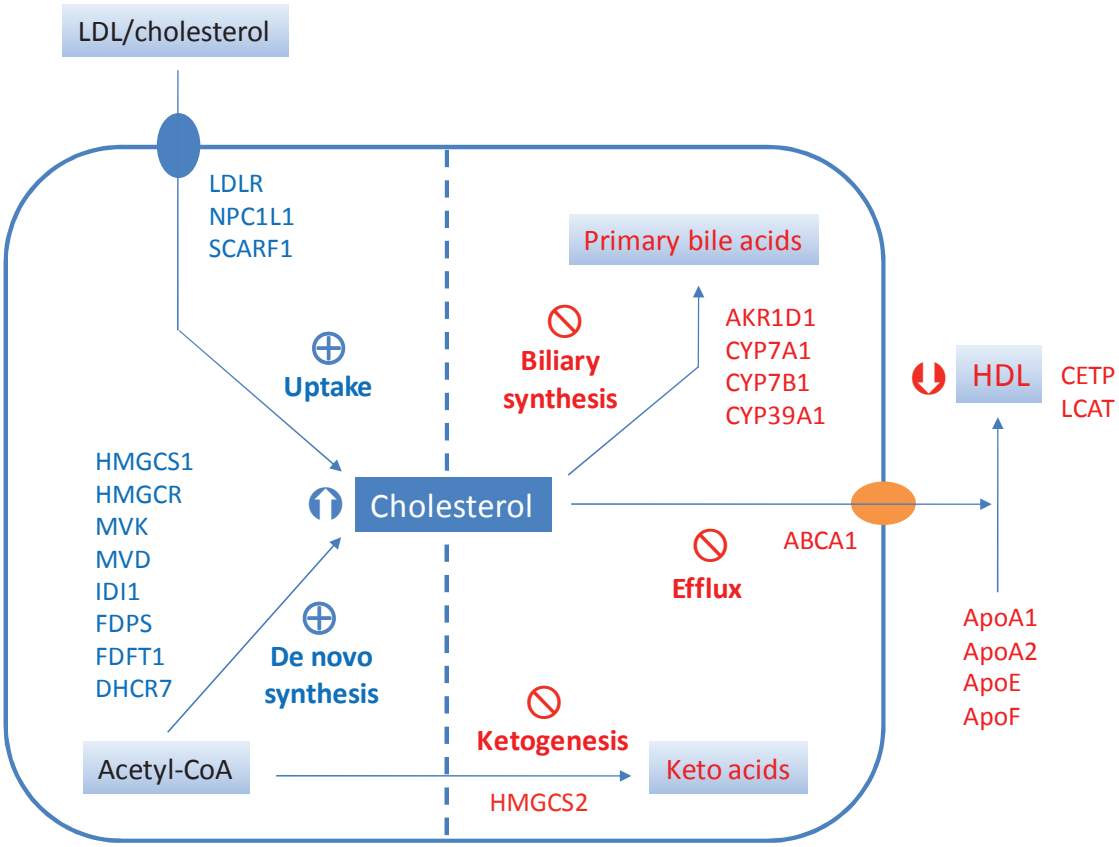

Figure 2. Exposure of HepG2 cells to different alkylphospholipids deregulates the expression of specific genes involved in cholesterol transport and metabolism.

4.4. Alkylphospholipids deregulates cholesterol metabolism and induce cell cycle arrest and autophagy in U-87 MG glioblastoma cells

In this work, we extended our study to a different tumor cell line of that probed in previous studies being human glioblastoma cell line U-87 MG chosen as the experimental model. It is remarkable that glioblastoma is the most frequent primary malignant brain tumour in adult humans and median survival is generally less that 1 year from the time of diagnosis. Thus, in this work we deep on cholesterol accumulation caused by APLs and its location inside cells through fluorescent microscopy. Moreover, morphologic alterations caused by APLs are also analyzed by transmission electronic microscopy (TEM).

Firstly, we compared APLs´ antiproliferative activity in U-87 MG cell line, obtaining a similar pattern to that of HepG2, but interestingly this cell line resulted to be quite more sensitive to all APLs since IC_{50} values were approximately half than obtained in HepG2. The analysis of cell division by flow cytometry shows that cell cycle is arrested in G2/M phase after APL treatments. Using TEM we observed that APLs provoked marked ultraestructural alterations related to autophagic processes. Coincidently with previous results, all APLs had inhibitory effect on cholesterol transport from plasma membrane to ER and increased cholesterol de novo synthesis in the glioblastoma cells.

Cholesterol accumulation caused in glioblastoma cell line was studied using the fluorescent substrate filipin, which binds to unesterified cholesterol and is detected under fluorescent microscopy. In this study we observed an increase of filipin´s intensity inside cells after APL-treatment. Differences were found in filipin/cholesterol distribution between control and treated cells since in those, cholesterol was localized in plasma membrane and perinuclear regions whereas APL-treated cells had a more diffuse pattern mainly inside the cells. It has been reported that in some lipid accumulation diseases (e.g. Niemann Pick C disease, NPC) cells present a phenotype consisting in punctuated filipin staining inside cells that colocalize with late endosome/ lysosome (LE/LY) compartment. Very interestingly in this study, APL- treated cells did not shown cholesterol colocation with LE/LY compartment, indicating that this cholesterol accumulation is not probably related with endocytic pathway.

Thus, the findings of this study clearly demonstrate that the effects of antitumoral APLs are result of a complex mechanism involving cholesterol homeostasis disruption, autophagy and cell cycle arrest. The knowledge of the interrelationships between these processes is fundamental to understand tumoral response and will facilitate the development of novel therapeutics to improve interventions in glioblastoma and other types of cancer.

Alkylphospholipids deregulate cholesterol metabolism and induce cell-cycle arrest and autophagy in U-87 MG glioblastoma cells

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Abstract: Glioblastoma is the most common malignant primary brain tumour in adults and one of the most lethal of all cancers. Growing evidence suggests that human tumours undergo abnormal lipid metabolism, characterized by an alteration in the mechanisms that regulate cholesterol homeostasis. We have investigated the effect that different antitumoural alkylphospholipids (APLs) exert upon cholesterol metabolism in the U-87 MG glioblastoma cell line. APLs altered cholesterol homeostasis by interfering with its transport from the plasma membrane to the endoplasmic reticulum (ER), thus hindering its esterification. At the same time they stimulated the synthesis of cholesterol from radiolabelled acetate and its internalization from low-density lipoproteins (LDLs). Fluorescent microscopy revealed that these effects promoted the accumulation of intracellular cholesterol. Filipin staining demonstrated that this accumulation was not confined to the late endosome/lysosome (LE/LY) compartment since it did not colocalize with LAMP2 lysosomal marker. Furthermore, APLs inhibited cell growth, producing arrest at the G2/M phase. We also used transmission electron microscopy (TEM) to investigate ultrastructural alterations induced by APLs and found an abundant presence of autophagic vesicles and autolysosomes in treated cells, indicating the induction of autophagy. Thus our findings clearly demonstrate that antitumoural APLs interfere with the proliferation of the glioblastoma cell line via a complex mechanism involving cholesterol metabolism, cell-cycle arrest and autophagy. A knowledge of the interrelationship between these processes is fundamental to our understanding of tumoural response and may facilitate the development of novel therapeutics to improve treatment of glioblastoma and other types of cancer.

Keywords: alkylphospholipids, cholesterol homeostasis, cell-cycle arrest, autophagy, U-87 MG cells.

Abbreviations: APL, alkylphospholipid; ER, endoplasmic reticulum; LDL, low-density lipoprotein; LE/LY, late endosome/lysosome; TEM, transmission electron microscopy; LDLR, low-density lipoprotein receptor; ACAT, acyl-CoA:cholesterol acyltransferase; CE, cholesteryl esters; ErPC, erucylphosphocholine; HePC, hexadecylphosphocholine; LDH, lactate dehydrogenase; SREBP, sterol-regulatory element-binding protein; NPC1, Niemann-Pick Type C-1; NPC2, Niemann-Pick Type C-2.

Introduction

Brain tumours are the most malignant of cancers, posing major health problems and presenting especially difficult challenges to therapy. Among these, glioblastoma, a grade IV glioma, is the most frequent primary malignant brain tumour in adults, accounting for approximately 75% of all high-grade gliomas. Median survival rate is generally less that 1 year from the time of diagnosis, and even in the most favourable situations patients die within 2 years [15]. Thus glioblastoma is the most aggressively malignant glial primary brain tumour in humans and, being among the most chemo- and radiation-resistant types of cancers, one of the most lethal [18,21].

It has been reported that both human tumour and tumoural cell lines undergo abnormal cholesterol metabolism. In fact it is now recognised that the deregulation of cholesterol metabolism found in tumour cells is caused by an alteration in the mechanisms that form the basis of cholesterol homeostasis, particularly cholesterol synthesis, uptake of exogenous cholesterol by way of the low-density lipoprotein receptor (LDLR), cholesterol esterification mediated by acyl-CoA:cholesterol acyltransferase (ACAT) activity and cholesterol efflux [62]. This imbalance in cholesterol metabolism is reflected in an increase in the quantity of intracellular cholesteryl esters (CE) stored in lipid droplets in comparison with normal cells, purported to be due to a high esterification rate of intracellular cholesterol by ACAT activity. Thus, Nygren et al. [45] have reported increased levels of CE in glioma tissues and in surrounding infiltrated areas in the human brain. The presence of CE in malignant cerebral tumours may be related to neoplastic vascular and/or cell proliferation in the tumour mass, two prerequisites for tumour cell growth [61]. Pathways involved in CE accumulation seem in fact to be essential to tumour proliferation since inhibitors of cholesterol esterification cause a marked reduction in proliferation in leukaemia [44] and glioblastoma cell lines [3].

Alkylphospholipid (APL) analogues are a new class of antitumoural agents that do not target DNA but insert themselves into the plasma membrane and subsequently trigger a broad range of biological effects, which ultimately lead to cell death [49,59]. We have reported previously that some antitumoural APLs, such as perifosine, edelfosine, erucylphosphocholine (ErPC) and hexadecylphosphocholine

(HePC), share a common active mechanism that impedes the arrival of cholesterol to the endoplasmic reticulum (ER), thence producing a marked reduction in intracellular cholesterol esterification in HepG2 cells [8,53]. Consequently, exposure of these cells to APLs leads to a deregulation of cholesterol homeostasis. In our present work we have extended our previous studies to investigate the underlying mechanisms and consequences of the exposure of U-87 MG cells to APLs. Here we demonstrate that all the APLs tested block cholesterol esterification in this glioblastoma cell line whilst its synthesis and internalization increases, LDLR being up-regulated. Both direct assay and filipin staining reveal that intracellular free-cholesterol levels are enhanced. We also show that the APL treatments assayed cause cell-cycle arrest at G2/M by impeding exit from mitosis and that all these changes are accompanied by autophagy.

Materials and methods

Materials

Foetal bovine serum (FBS) was from The Cell Culture Company (Pasching, Austria), Minimal essential medium (MEM), TLC plates and Fluoromount from Sigma-Aldrich (Madrid, Spain), [1,2-^{3}H(N)]cholesterol and [1,2-^{14}C]acetate from Perkin Elmer (Massachusetts, USA), HePC from Cayman Chemical (Ann Arbor, USA), edelfosine from Calbiochem (Nottingham, UK), ErPC from Alexis Biochemicals (Exeter, UK) and perifosine from Selleck Chemicals (Ontario, Canada). Polyclonal anti-human LDLR and HRP-conjugated secondary IgGs were from Santa Cruz Biotechnology (Santa Cruz, USA), monoclonal primary antibodies (anti-human clathrin and anti-human P-cdc2) and secondary anti-rabbit Alexa Fluor 488 from Cell Signaling (Danvers, USA), LAMP2 monoclonal primary antibody from SouthernBiotech (Birmingham, USA) and secondary anti-mouse Alexa Fluor 568 antibody from Life Technologies (Madrid, Spain).

U-87 MG cell culture

The U-87 MG human glioblastoma cell line was from the European Collection of Animal Cell Cultures (Salisbury, UK). The cells were cultured in MEM containing 10% heat-inactivated FBS supplemented with 2 mM L-glutamine, 1% non-essential

amino acids, 100 U/mL penicillin and 100 µg/mL streptomycin, in a humid atmosphere with 5% CO_2 at 37ºC, and subcultured at a ratio of 1:10 once a week.

Assays for cell proliferation and morphology

U-87 MG cells were seeded onto 96-well plates (10,000 cells/well) and maintained in MEM containing 10% FBS for 24 h. The culture medium was then replaced with fresh MEM/10% FBS and the cells incubated for 24 h in the absence or presence of different quantities of APLs. The antiproliferative effect of the compounds was assessed by crystal-violet staining using a cell-number-based standard curve. To this end the medium was removed and the cells fixed by the addition of 100 µl of 1% glutaraldehyde in PBS solution. After orbital shaking (50 rpm for 15 min) the plates were washed eight times with ultrapure water, air-dried and stained by the addition of 200 µl of a 0.1% solution of crystal violet dissolved in water. After orbital shaking (30 rpm for 20 min) the dye was removed and the plates washed thoroughly with deionized water. The samples were air-dried prior to dye solubilisation in 100 µl of 10% acetic acid. The absorbance of crystal violet in each well was measured directly in the plates at a wavelength of 590 nm using an ELx800 microplate reader (Bio-tech Instruments, Inc, Spain).

The cytotoxicity indexes used were lactate dehydrogenase (LDH) leakage and change in cell morphology, observed under an inverted Olympus CK2 microscope (Olympus, Spain).

Transmission electron microscopy

U-87 MG cells were plated in six-well dishes and allowed to grow for 24 h. Subsequently, either 10 µM HePC or ErPC, or just a vehicle as control, was added for 24 h. Cells were collected using trypsin and centrifuged at 1,500 rpm for 5 min in MEM/10% FBS. Cell pellets were fixed in 2.5% glutaraldehyde + 2% paraformaldehyde in 0.05 M cacodylate buffer for 4 h at 4ºC. The samples were washed three times with cacodylate buffer. Postfixation was done in an aqueous solution of 1% OsO_4 containing 1% potassium ferrocyanide for 1 h at 4ºC in darkness. The following washes were done: 0.15% tannic acid in cacodylate buffer, cacodylate buffer, and water, all at room temperature (rt). The samples were left in 2% uranyl acetate for 2 h and washed several times in water before being dehydrated at 4ºC in

ethanol solutions rising from 50% to 100%. The samples were put into resin [EMbed 812/100% ethanol (1/1)] for 60 min at rt, the same resin at a 2/1 ratio for 60 min, and then resin without ethanol overnight. For polymerization, the samples were incubated in pure resin for 48 h at 60ºC. Ultrafine sections (50-70 nm) were cut using a Leica Ultramicrotome R and contrasted using 1% aqueous uranyl acetate for 5 min and lead citrate in a CO_2-depleted atmosphere for 4 min [52]. A Zeiss Libra Plus 120 electron microscope was used to study the sections.

Flow-cytometry analysis

U-87 MG cells were plated in six-well dishes at a seeding density of 5×10^4 cells/well. After 24 h the medium was removed, the cells were washed three times with PBS and incubated in MEM containing 0.04% FBS for 24 h for cell synchronization in the G0/G1 phase. The low-serum medium was removed and the cells were then incubated in the presence of APLs (5-25 μM) or vinblastine sulphate (2.5 mg/ml) in a medium containing 10% FBS. After 24 hours' exposure the cells were harvested by trypsinization, collected, fixed with 1% formaldehyde, treated with 70% ethanol to solubilise their membranes and then stained with 5 μg/ml propidium iodide in the presence of 200 μg/ml of RNAse A and kept in the dark. The stained cells were incubated at rt for 30 min before being analysed by flow cytometry using a Becton Dickinson FACS Vantage.

Trafficking of cholesterol from the plasma membrane to the endoplasmic reticulum

U-87 MG cells were seeded in 12-well plates at 60-70% confluency and incubated with 2 μCi/ml of [1,2-^{3}H(N)]cholesterol for 60 min at 37ºC in MEM. To remove any unincorporated radioactivity the cells were washed three times with PBS containing 0.5% BSA, and twice with PBS (both pre-warmed to 37ºC). The cells were then incubated for 1 h at 37ºC in MEM/10% FBS in the absence or presence of HePC, edelfosine, ErPC or perifosine, as described by Marco et al. [36]. Subsequently the medium was removed, the cells washed with PBS and the lipids extracted following the procedure of Bligh and Dyer [4]. Cholesterol and CE were separated by TLC using a mixture of hexane:diethyl ether:acetic acid (70:30:2) as solvent. Radiometric measurements of scraped lipid spots, rendered visible by exposure to iodine vapour,

were made by liquid scintillation. The fraction of plasma-membrane cholesterol esterified in the ER is expressed in terms of the percentage of esterification of total labelled cholesterol.

Cell uptake of [1,2-^{3}H(N)]cholesterol in LDL

[1,2-^{3}H(N)]cholesterol was incorporated into LDL using a modification of the procedure described by Sugii et al. [60]. Briefly, 40 μCi (0.74 nmol) of [1,2-^{3}H(N)]cholesterol was evaporated to dryness. The residue was dissolved in 0.1 ml of dimethyl sulphoxide and the clear solution was shaken on a vortex mixer. Subsequently 0.9 ml of a buffer containing PBS/1mM EDTA (pH 7.5) was added, followed by 1 mg of LDL. This solution was incubated for 2 h at 40ºC, after which it was dialysed at 4ºC for 36 h against four changes of 4 l of buffer consisting of PBS/1mM EDTA (pH 7.5). The solution was then removed from the dialysis tubing and centrifuged at 12,000 rpm for 5 min. The dialysed solution was stored at 4ºC until required (1 week maximum). For the assay, U-87 MG cells at 60% confluence were incubated with vehicle, 10 μM APLs or 1 μM U18666A, for 24 h in MEM/10% FBS. [1,2-^{3}H(N)]cholesterol-LDL was added for the last 6 h of incubation, after which the medium was removed, the lipids extracted, the cholesterol and CE separated and radiometric measures undertaken as described above.

Cholesterol biosynthesis assays

Cells were seeded in 12-well plates at 60-70% confluency. Vehicle or 10 μM APL was added for 24 h. The medium was then renewed and the cells labelled with 1 μCi/ml [1,2-^{14}C]acetic acid in MEM/10% FBS for 2 h. The medium was subsequently removed and the cells washed 3 or 4 times with PBS. Lipids were extracted, cholesterol and CE separated and radiometric measurements undertaken as described above. Some aliquots were taken before TLC and a commercial kit (Randox, UK) was used to determine total cholesterol levels in the samples.

Inmunoblotting analysis

U-87 MG cells growing in log-phase were incubated for 24 h with MEM/10% FBS in the absence (PBS as vehicle) or presence of APLs. The cells were washed twice, scraped into ice-cold PBS (pH 7.4) and centrifuged at 100 x g for 10 min at

4°C. Cell pellets were suspended in 0.3 ml ice-cold lysis buffer consisting of 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1% Triton X-100 and a protease inhibitor cocktail (Sigma, Madrid, Spain) and incubated on ice for 30 min with occasional shaking. Cell lysates were centrifuged at 10,000 x g for 15 min at 4°C and supernatants were stored at -80°C until use; an aliquot was taken to determine protein concentration. Equal quantities of lysate protein were separated by 10% SDS-PAGE and transferred onto polyvinylidene difluoride membranes. Prestained broad-range protein molecular-mass markers were used during electrophoresis. Membranes were blocked in TBS containing 5% non-fat dried milk and 0.05% Tween-20 for 1 h, and then probed with anti-human primary Igs (1:1,000) in blocking solution for 1 h at 4ºC overnight. After several washes in TBS containing 0.05% Tween-20, the membranes were incubated with the corresponding horseradish peroxidase (HRP)-conjugated IgG (1:5,000) as secondary antibody for 1 h. Immunoreactive proteins were detected by autoradiography using a chemiluminescent HRP substrate and exposure to Konica Minolta X-ray film (Tokyo, Japan). Following incubation with an antibody-stripping solution consisting of 60 mM Tris-HCl (pH 6.8), 100 mM β-mercaptoethanol and 2% SDS for 30 min at 60°C, blots were probed with rabbit polyclonal anti-human β-actin Ig (1:1,000) to monitor the loading and transfer of the blotted samples. Densitometric analysis was carried out using ImageJ gel-digitizing software from the National Institutes of Health (Bethesda, MD, USA).

Filipin staining

U-87 MG cells were seeded in microscopy slides at 5,000 cells/chamber and allowed to adhere and grow for 24 h. Cells were incubated with vehicle, 25 μM APLs or 1 μM U18666A for 24 h, fixed in 4% p-formaldehyde for 30 min at rt and washed 2 or 3 times with PBS. To prevent photobleaching, 50 mM Cl_4Mg_2 was added for 10 min. Cells were incubated at 37ºC with 500 μg/ml of filipin in PBS/10% FBS, which permeates the cell and specifically stains free cholesterol [47].

For colocalization studies the cells were stained with filipin, washed with PBS and blocked with PBS/10% FBS for 1 h at rt. They were incubated overnight at 4ºC with PBS/10% FBS containing primary antibodies at 1:50 and then rinsed three times in PBS/Tween before being incubated with fluorescent secondary antibodies (Alexa 488 or Alexa 568) for 60 min (1:500) and finally washed once more. Chamber walls

were removed and slides were mounted using Fluoromount. The slides were studied under a LEICA DM5500B fluorescent microscope. Filipin intensity was measured cell by cell (30-50 cells/sample) using Image J software.

Statistics

Results are expressed as means ± SEM. A one-way ANOVA with *post hoc* comparisons by Scheffe´s test was carried out (SPSS 13.0). $P < 0.05$ was considered to be statistically significant.

Other analyses

Cell-protein content was determined in the cell homogenates by Bradford´s method [5] using BSA as standard.

Results

Alkylphospholipids inhibit cell growth in human glioblastoma cells

Proliferation of U-87 MG cells following treatment with various concentrations of APLs was assessed by crystal-violet staining. Incubation with 1-25 μM APLs for 24 h led to a dose-dependent decrease in cell growth (Fig. 1A). The IC_{50} values derived from the growth inhibition curves were 7.11 ± 1.38 μM for edelfosine, 19.04 ± 1.53 μM for perifosine, 22.14 ± 0.97 μM for ErPC and 36.91± 8.72 μM for HePC. Thus the inhibitory potency of the four APLs assayed shows the following pattern: edelfosine > perifosine ≈ ErPC > HePC. We did not detect LDH activity in the culture medium after any of the treatments up to 25 μM (data not shown) and so the decrease observed after 24 hours' treatment could not be attributed to any acute cytotoxicity produced by plasma-membrane leakage. The morphology of the cells, observed under an inverted microscope after 6 and 24 hours' APL treatment, showed alterations such as condensation and rounding, both being traits of cell damage (Fig. 1B).

Ultrastructural alterations produced by alkylphospholipids

We used TEM to examine the ultrastructure of U87-MG cells exposed to ErPC and HePC as representative APLs. Alterations to their ultrastructure are shown in Figure 2, where it is clear that the morphology of the cells in the control group is normal (Fig. 2A), characterized by fine-textured nuclear chromatin and intact nuclear and cytoplasmic membranes. Their rough ER and Golgi apparatus profiles are also normal. Mitochondria are relatively abundant and easily distinguishable, with a dense matrix and well defined cristae. Vacuolar structures, resembling autolysomes, are poorly developed.

Both ErPC and HePC treatments, on the other hand, resulted in significant alterations to cell ultrastructure, although the effects were markedly more patent in ErPC-treated samples. The most striking feature provoked by HePC (Fig. 2B and C) and ErPC (Fig. 2D, E and F) was the increase in the number of cytoplasmic-membrane-limited vesicles containing fragments of rough ER, membranous structures, and several lamellar formations that appear to correspond to autophagic vesicles engulfing cytoplasm and cell organelles. In fact micrographs (Fig. 2G to K) reveal structures that reflect various steps in the process of autophagy, from double-membraned vesicles containing recently engulfed organelles to residual bodies, these latter being characteristic of the final step of the autophagic process. Also noteworthy is the presence of single-membraned vesicles containing electron-dense elements and partially degraded material, probably corresponding to autolysomes produced by the fusion of autophagosomes with lysosomes, together with some multivesicular bodies. Interestingly, in the ErPC-treated cells some open autolysosomes can be seen to have expelled their content, suggesting that the lytic process has begun to affect the surrounding cytoplasm.

Neither the control nor the treated cells show any morphological evidence suggestive of apoptosis, such as nuclear shrinkage, margination and condensation of the chromatin, enlargement of ER cisternae or convolution of the cell with the formation of apoptotic bodies.

Alkylphospholipids affect the distribution of the cell-cycle phases in human glioblastoma cells

To examine whether APL-induced cytotoxicity was associated with cell-cycle deregulation, cell-cycle distribution was analysed by flow cytometry. In an initial assay, dose-response was studied by treating cells with increasing concentrations of APLs. As illustrated in Figure 3A, the exposure of glioma cells to 10 μM of ErPC or perifosine (as representative APLs) resulted in an accumulation of glioblastoma cells in the G2/M phase, rising significantly from 15.57% in the solvent-treated controls to 21.54% in those treated with ErPC and 21.81% with perifosine. Vinblastine sulphate, which has been shown to increase the percentage of many cell lines in the G2/M phase [57], was used as positive control.

Cell-cycle distribution after APL treatment is set out in Table 1. In control U-87 MG cells, 52.50 ± 0.69% cells were found to be in the G0/G1 phase, 16.19 ± 0.42% in the G2/M phase and 29.27 ± 0.73% in the S phase of the cell cycle 24 h after plating. As can be seen, APLs increased the percentage of cells in the G2/M phase significantly, thus arresting the proliferation of glioblastoma cells during this phase.

The levels of some proteins involved in G2/M transition were further examined by Western blotting. The phosphorylation status of cdc2 kinase governs the activity of the cdc2/cyclin B1 complex, which is responsible for the onset of mitosis [21,46]. As can be seen in Figure 3B, the exposure of U-87 MG cells to APLs decreased the phosphorylation status of Tyr15 in cdc2 kinase protein. In spite of the fact that p21 has been implicated in the control of the G2/M checkpoint [6], APL treatment did not significantly affect p21 expression, with only very small variations being detected (data not shown).

Alkylphospholipids interfere with cholesterol traffic from the plasma membrane to the endoplasmic reticulum

To ascertain whether APLs affect cholesterol metabolism in U-87 MG cells we also studied their effects on the traffic of cholesterol from the plasma membrane to the ER. In the belief that the best experimental procedure to study any interference in this route is to analyse the rate of cholesterol ester synthesis from plasma-membrane

cholesterol [26,36] we labelled plasma membrane with radioactive cholesterol for 60 min before exposing it to the different APLs in the presence of MEM/10% FBS and determining the radioactivity appearing in the esterified cholesterol. We also tested the effect of U18666A compound, a hydrophobic amine extensively used to study cholesterol homeostasis, and more particularly intracellular cholesterol trafficking [10], which has been reported to promote cholesterol intracellular accumulation, occurring as it does in some diseases involving lipid disorders [38]. Figure 4 shows how both U18666A and APL treatments led to a decrease in CE formation, thus demonstrating an inhibition of cholesterol transport from the plasma membrane to ER.

Filipin staining pattern is altered by alkylphospholipids

We labelled cholesterol with filipin, a fluorescent compound that creates pores in fixed cells and binds to free cholesterol molecules [24,47], so as to be able to detect the cholesterol by fluorescent microscopy. We fixed and stained U-87 MG cells as described in Materials and methods. Interestingly, despite the inhibition observed in cholesterol trafficking (Fig. 4), fluorescence intensity was from 2 to 4.5 times higher in treated cells than in untreated ones (Fig. 5G), which concurred with total cholesterol determinations using a commercial kit. The control samples contained 40 μg cholesterol/mg protein, whilst total cholesterol levels in APL-treated cells rose to 60-100 μg/mg protein (data not shown).

With regard to the cholesterol distribution pattern, we can detect free-cholesterol staining in the plasma membrane and perinuclear region in U-87 MG control cells. Cytoplasm staining in APL-treated cells is more intense however, indicating that the cholesterol transport disruption described above does not produce an accumulation at plasma membrane level. It is interesting to note that, although filipin fluorescence intensity increases in a similar way in U18666A- and APL-treated cells, they show different patterns of cholesterol accumulation (Fig. 5B compared to C-F). With the former, cholesterol accumulates throughout the cytoplasm in a punctuated, vesicle-like pattern, whilst with APLs it is spread more diffusely. This indicates that cholesterol accumulation induced by U18666A and APLs could take place in different cell compartments.

Cholesterol uptake and biosynthesis are involved in cholesterol accumulation induced by alkylphospholipids

Increased cholesterol levels in cells may be due in part to an increase in LDLR-dependent uptake and/or cholesterol biosynthesis. For this reason we conducted metabolic assays to try to detect any possible alteration in either pathway. To determine whether APLs promote LDL uptake in U-87 MG cells we measured the internalization of radiolabelled cholesterol in LDL. Our results show that cholesterol-LDL uptake increased very significantly after 24 hours' treatment with APLs and that U18666A treatment also led to a clear increase in the internalization of cholesterol-LDL (Fig. 6A). We also carried out Western blotting to LDLR and according to the above results the levels of this receptor increased after treatment with the representative APLs (Fig. 6C). In spite of this increased uptake of cholesterol-LDL, its esterification was lower after all the treatments than that observed in the control cells (Fig. 6B), which agrees with the results shown in Figure 4.

Labelling with [1,2-^{14}C]acetate allowed us to measure the rate of cholesterol biosynthesis after APL treatment. The incorporation of radiolabelled acetate into cholesterol was significantly enhanced in U-87 MG cells exposed to APLs for 24 h compared to untreated ones (Fig. 6D).

Cholesterol accumulation caused by alkylphospholipids is not localised in the lysosomal compartment

As pointed out above, the results obtained with fluorescent microscopy demonstrated that APLs promote the accumulation of cholesterol within the cell cytoplasm. To further investigate the location of this cholesterol we conducted inmunofluorescence assays for cholesterol and proteic markers of clathrin-coated pits and the LE/LY compartment (Fig. 7A and B). In control U-87 MG cells filipin staining was diffuse and visible predominantly in the plasma membrane and some perinuclear regions, with no apparent colocalization with clathrin or LAMP2, which are participants in endocytosis. U18666A, however, produced a punctuated cholesterol staining pattern that partially colocalized with LAMP2 but not with clathrin. Interestingly, APL-treated cells did not show any cholesterol colocalization with clathrin or the LE/LY compartment. All our results indicate that treatment with

U18666A and APLs probably impairs cholesterol transport in different ways. Since in APL-treated cells cholesterol accumulation is not located in the LE/LY compartment, cholesterol transport inhibition caused by APLs would seem to be independent of the endocytic pathway.

Discussion

The extent of the cytotoxic effects of APL derivatives depends upon the tumour cell line in question [63]. Few reports exist describing the effects of APLs on glioblastoma cells. Our results prove that the human glioblastoma cell line U-87 MG is more sensitive to APLs than are some other human cell lines [8], showing IC_{50} values of approximately 7 μM for edelfosine to approximately 37 μM for HePC. The antiproliferative action of the four APLs studied in our laboratory was unrelated to cytolysis since prolonged incubation of cells with doses of up to 25 μM led to no significant release of LDH from the cells into the culture medium.

Inhibition of cell growth may be related to an alteration in the progress of the cell cycle. Several studies have shown that some APLs induce cell-cycle arrest in several cell lines [9]. The main checkpoints that control the cell-division cycle in eukaryotes include: G1, G2 and the metaphase [16]. The second checkpoint is located at the end of the G2 phase, which triggers the start of the M phase (mitosis). The entry of eukaryotic cells into mitosis is regulated by cdc2 kinase activation, a process controlled at several steps, including cyclin binding and the phosphorylation of cdc2 at Thr161 [2]. Nevertheless, the critical regulatory step in activating cdc2 during progression into mitosis appears to be the dephosphorylation of cdc2 at Thr14 and Tyr15 [54] (Fig. 8).

We found that APLs were able to bring down the quantities of phospho-Tyr15 cdc2 kinase in this cell line, indicating that they promote the entry of cells into mitosis. We also observed, however, that APL treatment arrested U-87 MG at the G2/M stage. A similar effect has been reported with vinblastine, a well known drug used to treat certain kinds of cancer, and other drugs that provoke mitotic arrest and accumulation of cells at the G2/M phase [50,57]. From these results, we postulate that cells treated with APLs are probably able to initiate mitosis as their DNA remains undamaged, but

are unable to conclude the process. As far as this is concerned, a recent investigation has shown that impeding the final step of cell division effectively kills tumour cells *in vivo* and thus targeting the exit of mitosis has recently been proposed as a viable therapeutic approach against cancer [35].

Several studies conclude that cholesterol metabolism plays a relevant role in the decision making between cell proliferation and differentiation [30] and thus the great current interest in studying the role of cholesterol in tumour genesis and exploring the possibility of interfering with its metabolism to modulate the growth of cancer cells. Cholesterol metabolism is tightly regulated at the cell level by a complex set of mechanisms, including cholesterol biosynthesis, the hydrolysis of CE from lipoproteins internalized into lysosomes, and the transport of released cholesterol to intracellular organelles such as the ER for its subsequent esterification [55]. Key to this regulation is a family of membrane-bound transcription factors known as sterol-regulatory element-binding proteins (SREBPs). Under low-sterol–concentration conditions, active SREBPs are translocated to the nucleus, where they regulate the transcription of sterol-responsive genes involved in cholesterol synthesis (3-hydroxy-3-methyl-coenzymeA reductase) or uptake (LDLR) [39]. Another important factor in this tight regulation is the esterification of cholesterol by ACAT enzyme, located in the ER [11]. In response to an increased concentration of cholesterol in the ER, ACAT rapidly esterifies free cholesterol to be stored in cytoplasmic lipid droplets. Cholesterol esterification by ACAT is a mechanism used by cells to prevent the accumulation of free cholesterol and its conversion into oxysterols [22,56]. Increased production of CE, and ACAT expression and activation have been measured in different human tumours and a greater capacity to esterify and accumulate cholesterol in tumours cells has in fact been associated with higher growth rate, suggesting a link between CE production and cell proliferation [37,61].

Cells obtain cholesterol by taking it up from their environment, mostly in the form of LDL. In fact, receptor-mediated endocytosis of lipoproteins such as LDL and the hydrolysis of their CE cores in the LE/LY compartment constitute a major source of cell cholesterol. Since sterols are not particularly abundant in LE/LY the processed cholesterol must escape rapidly from these compartments. How LDL-derived sterols are trafficked out of the LE/LY to reach the plasma membrane and possibly other

organelles is not completely understood, though it is known that the mechanism involves at least two key proteins that reside in the LE/LY: Niemann-Pick Type C-1 (NPC1) and C-2 (NPC2) proteins [34]. Indeed, mutations of either of these proteins lead to the retention of cholesterol and other lipids in these organelles, and such defects are the basis of the inherited autosomal recessive NPC disease [38,40,43]. The NPC cell phenotype involves the formation of LE/LY-like storage organelles, which are sterol sinks not only for LDL-derived sterol but also for sterols coming from the plasma membrane and other membranes [43]. Interestingly, the NPC cell phenotype can be mimicked by treating normal cells with various amphiphilic compounds such as the steroid U18666A, which has been shown to inhibit the esterification of plasma-membrane cholesterol and to induce cholesterol biosynthesis [12,23,29].

Bearing all this in mind, it is evident that the accumulation of free cholesterol produced in U-87 MG cells after APL treatment should down-regulate cholesterogenic enzymes and LDLR, thus preventing both cholesterol synthesis and uptake. On the contrary, both processes are visibly increased after APL exposure, clearly pointing to the fact that APLs induce a deregulation of cholesterol metabolism. Thus the results of our study demonstrate that APLs increase LDL-cholesterol incorporation and decrease cholesterol esterification in U-87 MG cells. All these results imply that the normally stringent sterol-dependent feedback regulation of cholesterol synthesis and uptake is disrupted by APLs, resulting in substantial free-cholesterol accumulation.

Maintenance of the cycle between free and esterified cholesterol relies on the bidirectional transport of sterols between the ER and the plasma membrane and/or an endocytic compartment. Cholesterol travels from the plasma membrane to the ER via a different route from that taken by the nascent cholesterol departing from it [17,58]. Some amphiphilic drugs, such as U18666A, progesterone and imipramine, have been described as interfering with intracellular sterol traffic by accumulating lysosomal unesterified cholesterol in several cell lines [28,41]; the movement of cholesterol from the cell surface to the ER is inhibited by these drugs, which also affect cholesterol transport from the endosomes [27]. In fact, it has been reported that U18666A enhances filipin staining in different cell types, such as CHO [14,48], MEFs [20] and HepG2 [23] cells. This increase is part of the NPC-like phenotype that

has also been described as an accumulation of cholesterol in the LE/LY compartment and which results in a colocalization between filipin and lysosome proteins such as LAMP1 [51] or LAMP2 [1]. In our study we have confirmed the location of cholesterol in the LE/LY compartment after U18666A treatment because filipin and LAMP2 are colocalized after this treatment in U-87-MG cells. As far as clathrin is concerned, this is a molecular marker of the clathrin-coated pits produced in the process of endocytosis mediated by this protein. After a vesicle buds into the cytoplasm the coat rapidly disassembles, allowing the clathrin to recycle while the vesicle can become early endosomes [42]. The rapid recycling of clathrin is the most probable reason for there being no filipin-clathrin colocalization in positive control U18666A. Neither were we able to see cholesterol accumulation in the LE/LY in either HePC- or ErPC-treated cells, nor with perifosine or edelfosine (data not shown), suggesting that APL-induced traffic inhibition does not occur via the endocytic pathway.

As we have observed, the altered cholesterol traffic and metabolism were associated with enhanced autophagy, so the ultrastructural features seen in APL-treated cells indicate that the effect of these phospholipid analogues upon U87-MG cells was characterized by an abundant presence of autophagic vacuoles and autolysosomes. These structures are consistent with autophagy, as has been described elsewhere [7,25]. Autophagy is a dynamic process in which intracellular membrane structures, the so-called autophagosomes, sequester proteins and organelles for degradation [32]. In evolutionary terms it is a highly conserved process that occurs in all eukaryotic cells without exception, providing energy during periods of starvation. On the other hand, autophagy has recently been defined as a distinct type of cell death (so-called type-II programmed cell death) [13,19] and in fact several lines of evidence suggest that cross-talk exists between the pathways leading to autophagy and apoptosis [13]. Autophagy begins with double-membrane-bound structures, known as autophagosomes, engulfing cytosol and/or organelles. These autophagosomes finally fuse with lysosomes to form autolysosomes, which mediate the subsequent degradation and recycling of cell components. Accumulating evidence argues for an important role for autophagy in the development of cancer [31].

To our knowledge there have been no reports to date concerning a possible relationship between APLs and the induction of autophagy in cells, but nevertheless, recent studies do suggest that cholesterol accumulation in cells in pathophysiological states, such as altered cholesterol trafficking, may well promote autophagy [33,64], such as that which occurred in our study. Our data suggest that it might be a direct causal link between cholesterol accumulation and increased autophagy, but whether this autophagy affords protection for the tumoural cells against APLs or rather drives cell elimination throughout type-II cell death in U-87 MG cells exposed to APLs remains uncertain. Clearly further studies must be conducted to solve these questions.

In summary, our findings in this study clearly demonstrate that APLs interfere with processes vital for cell survival, such as cell growth, cholesterol metabolism, autophagy and the cell cycle itself. A knowledge of the interrelationships between these processes is fundamental to our understanding of tumoural response and should facilitate the development of novel therapeutics to improve the treatment of glioblastoma and other types of cancer, and should also be born in mind in clinical applications aimed at reducing glioma growth.

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Fig. 1.A

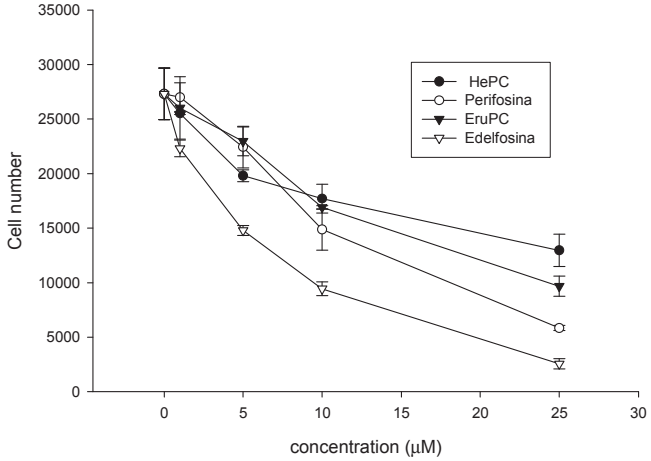


Fig. 1.B

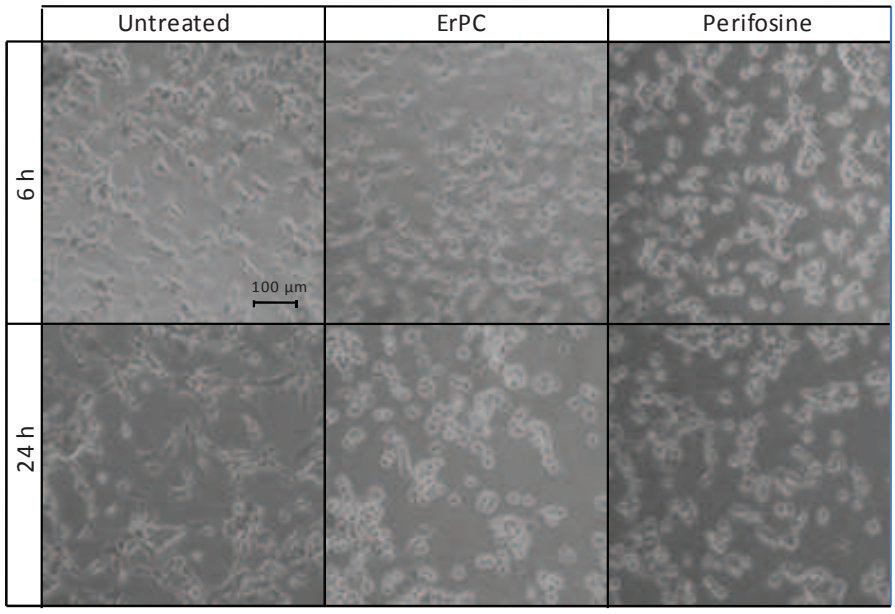


Figure 1. Effect of APLs on U-87 MG proliferation and morphology. U-87 MG cells growing in log-phase were incubated with MEM/10% FBS in the absence or presence of different concentrations of APLs for 24 h. Cell numbers were determined by crystal-violet staining and expressed as a percentage of the control cells (A). Cell morphology was examined under an inverted microscope. The morphology of the U-87 MG cells is shown in the absence (untreated) or presence of 25 μM of ErPC or perifosine for 6 h and 24 h (B).

Fig 2.

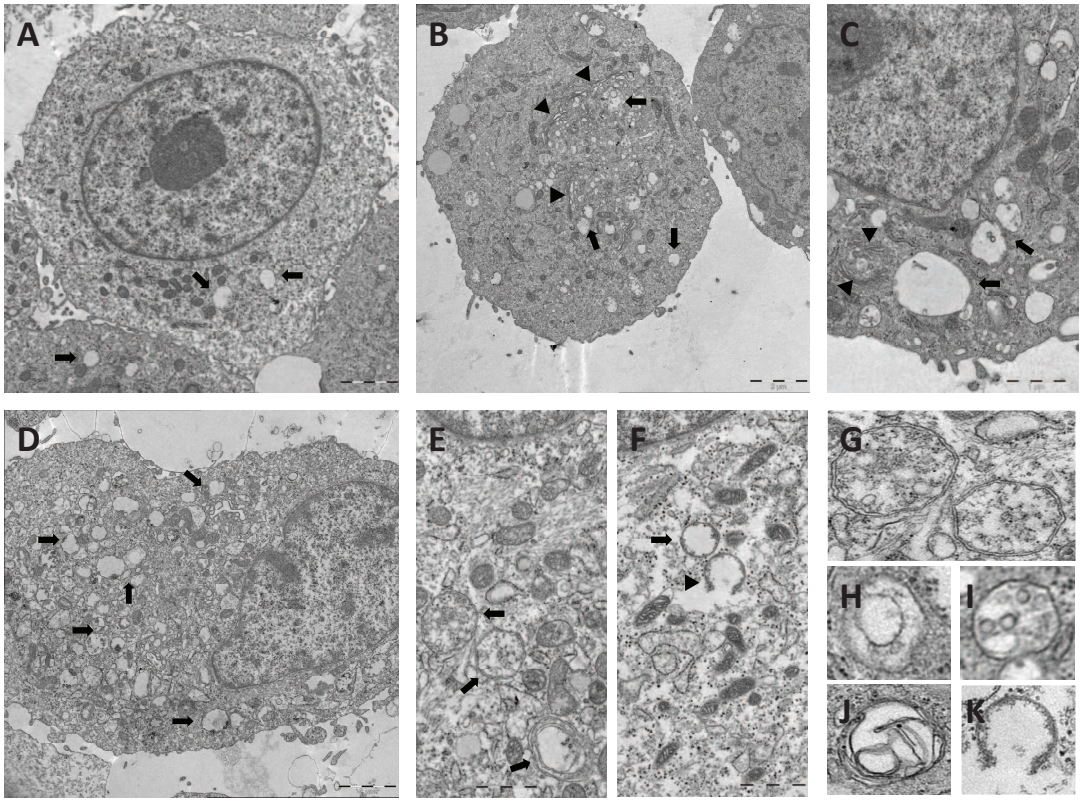


Figure 2. U-87 MG cells observed by TEM. U-87 MG cells growing in log-phase were treated with vehicle, 10 μM HePC or ErPC for 24 h. Cells were detached, collected and prepared for electron microscopy as explained in Materials and methods. A: U87-MG control cells showing a well preserved nucleus, dense mitochondria and well defined profiles of rough ER with scarce lysosomal vesicles (arrows); B and C: HePC-treated cells: numerous vesicles at different phases of the autophagic process (arrows) are visible in the cell cytoplasm together with many enlarged Golgy cisternae (arrowhead); D, E and F: General ultrastructure and details of cytoplasm of ErPC-treated cells; notable increases in vesicles and vacuoles (arrows) showing different stages of the autophagic process are evident; double-membraned vesicles, autophagosomes (G); autolysosomes (H); multivesicular bodies (I); and residual bodies with lamellar content (J); F and K: Some ruptured autolysosomes with intravesical content in communication with the cytoplasm (arrowhead) can be seen, suggesting that the autolytic process has extended to the cytoplasmic material.

Figure 3.A

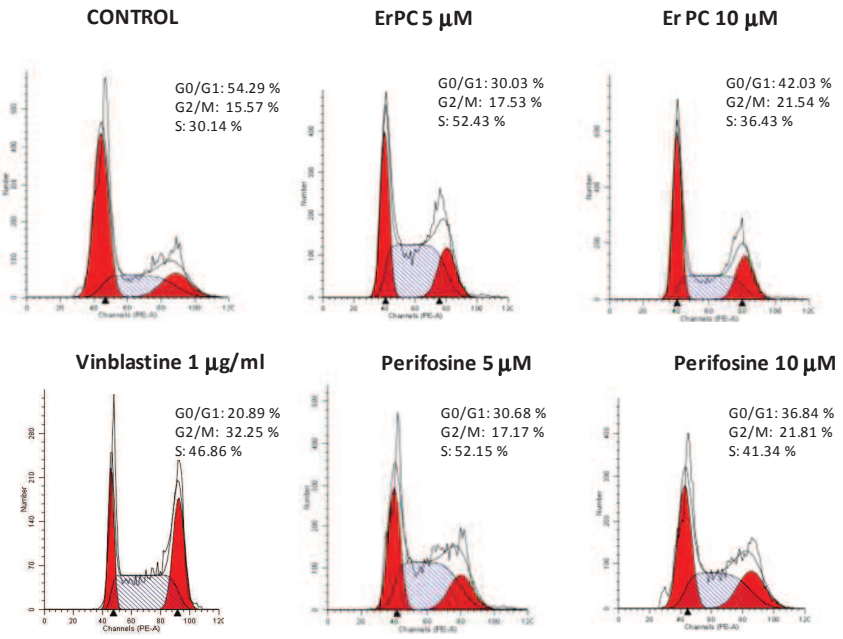


Figure 3.B

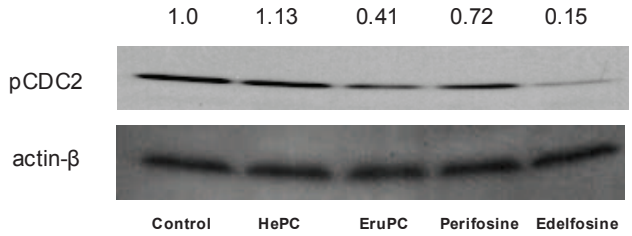


Figure 3. APLs arrest cell cycle at the G2/M phase. For cell synchronization, U-87 MG cells growing in log-phase were washed and incubated in a medium containing 0.04% FBS for 24 h. They were then incubated in the absence and presence of APLs (5-25 µM) or vinblastine sulphate (2.5 mg/ml) in a medium containing 10% FBS before being collected and submitted to flow cytometry as described in Materials and methods. Flow-cytometry histograms of the control and treated cells show that G2/S is increased in APL- and vinblastine-treated cells (A); Immunoblotting was done with APL-treated U-87 MG cell lysates (B). Synchronized cells were exposed to APLs in MEM/10% FBS for 24 h; SDS-PAGE and Western blotting for Tyr-15 phosphorylated Cdc2 was conducted as described in Materials and methods; pCdc2 protein levels in the samples were normalized to their respective β-actin level. Values are means of three independent experiments.

Fig. 4.

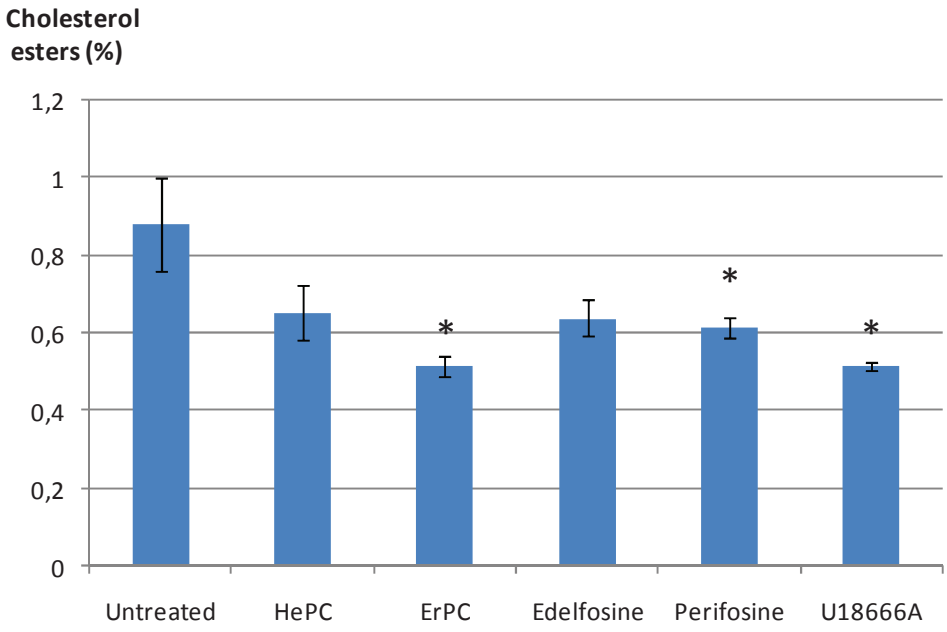


Figure 4. Transport of cholesterol from the plasma membrane to the endoplasmic reticulum. U-87 MG cells growing in log-phase were labelled with [1,2-^{3}H(N)]cholesterol for 60 min before being incubated with vehicle, 25 µM APLs or 1µM U18666A. Washes, lipid extraction, TLC and radiometric measures were done as described in Materials and methods. The fraction of esterified plasma-membrane cholesterol is expressed in terms of the percentage of esterification of the total labelled cholesterol and represents the mean of two independent experiments conducted in triplicate. *$P < 0.05$.

Fig. 5

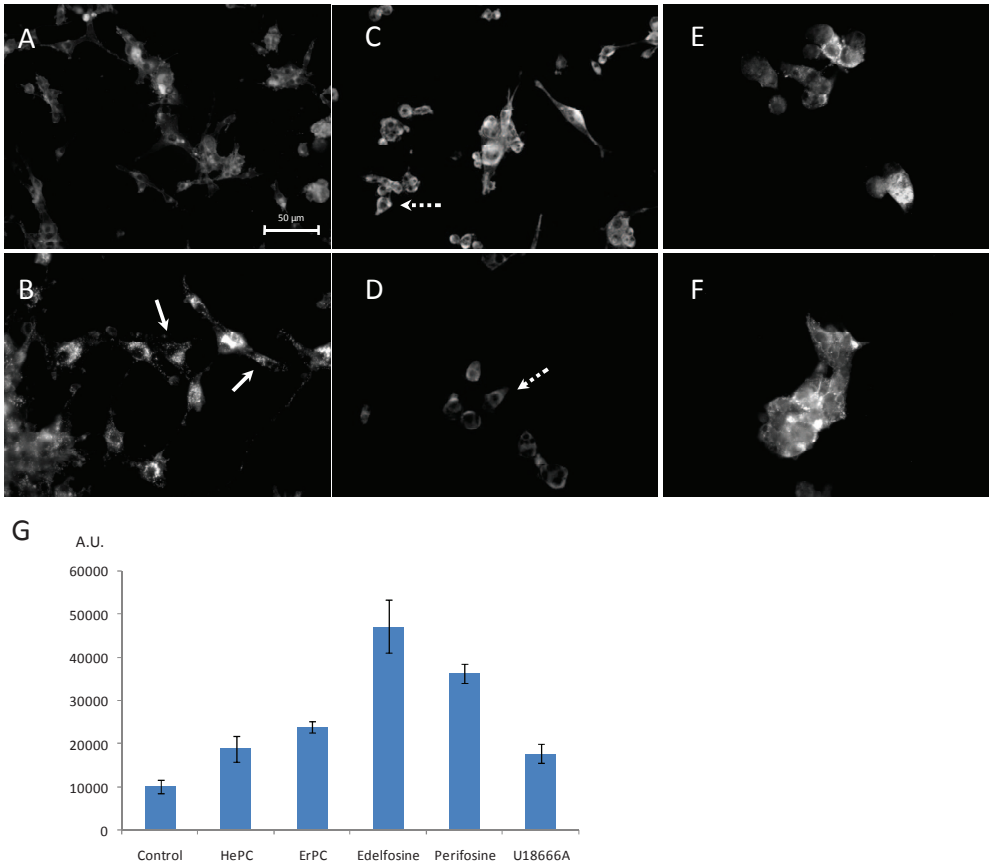


Figure 5. Filipin staining of U-87 MG cells. U-87 MG cells growing in log-phase were cultured over sterile coverslips and allowed to grow for 24 h. They were treated with vehicle (A), 1µM U18666A (B), 25 µM HePC (C), 25 µM ErPC (D), 25 µM edelfosine (E), 25 µM perifosine (F) for 24 h before being fixed and washed as described in Materials and methods. Filipin was added for 30 min. The cells were mounted using Fluoromount and visualized by fluorescence microscopy (A-F). A: Control cells showing cholesterol located in the plasma membrane and perinuclear region; B: U18666A treated cells showing a punctuated cholesterol cytoplasmic distribution (arrows); C to F: APL-treated cells showing cholesterol distributed diffusely (arrowhead): filipin intensity was measured using software Image J in 30-50 cells/treatment; G: Averages were calculated for control and treated cells and are represented and expressed as arbitrary units. These experiments were conducted three times.

Fig. 6.A

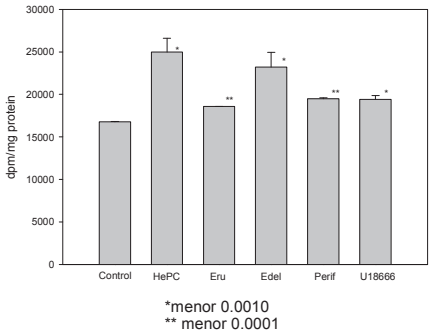


Fig. 6.C

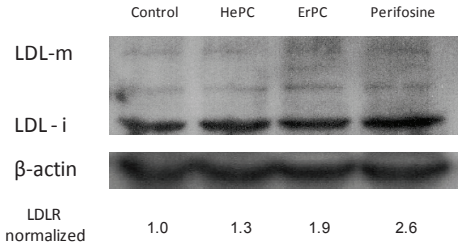


Fig. 6.B

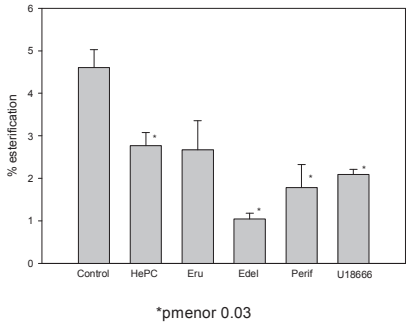


Fig. 6.D

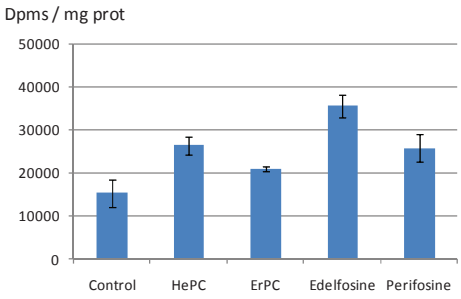
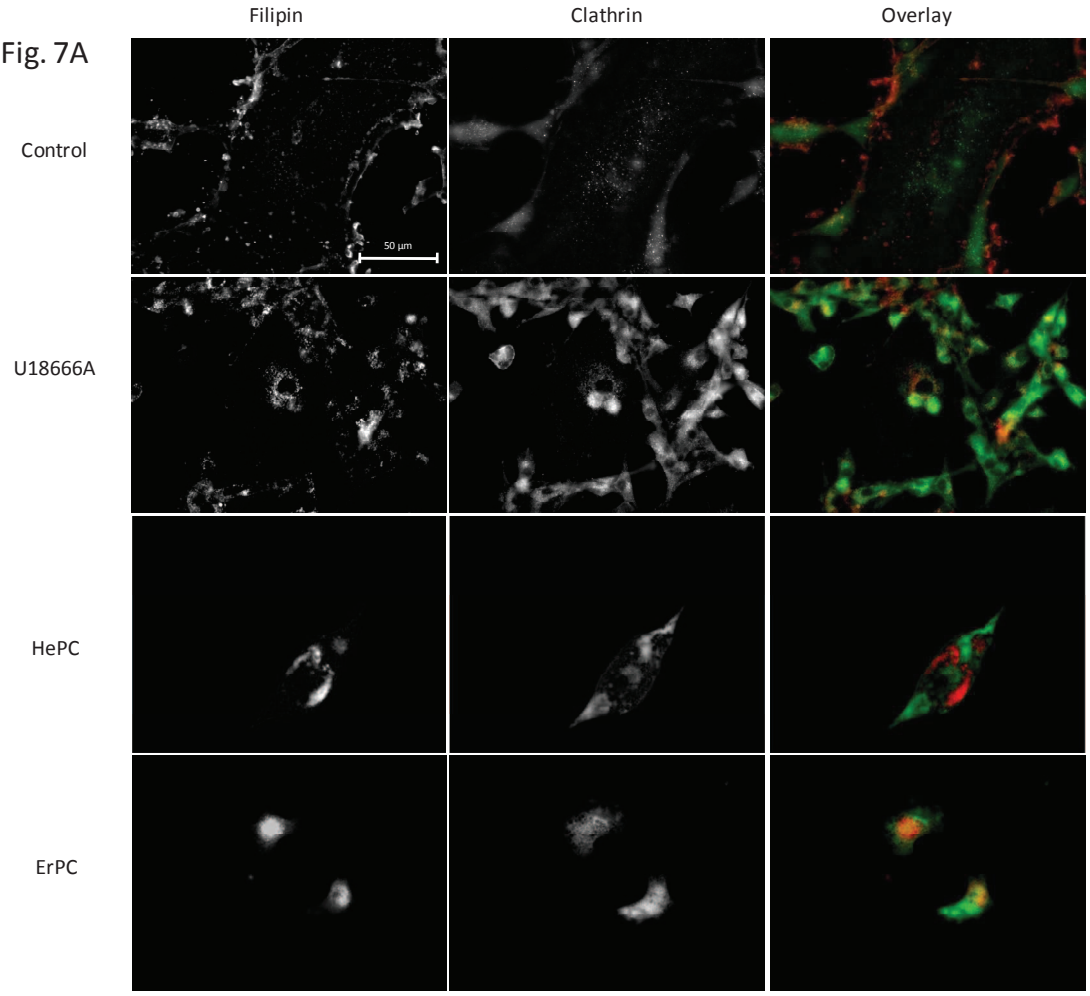


Figure 6. Effect of APLs on cholesterol uptake and biosynthesis. Cholesterol-LDL uptake (A) and cholesterol-LDL esterification (B) assays. U-87 MG cells growing in log-phase were incubated with vehicle, 10 µM APLs or 1 µM U18666A for 24 h in MEM/10% FBS. [1,2-^{3}H(N)]cholesterol-LDL was added for the last 6 h of incubation. A: Cholesterol-LDL uptake is expressed as internalized cholesterol (dpm/mg protein); B: Esterification percentage of internalized cholesterol; C: Western blotting of the LDLR in control and APL-treated cells; D: Cholesterol biosynthesis of control and treated U-87 MG cells was determined by the incorporation of [1,2-^{14}C] acetic acid into cholesterol. For the radiolabelling assays, lipid extraction and TLC were done as described in Materials and methods. These experiments were done twice. *P < 0.03; **P< 0.0001

Fig. 7A



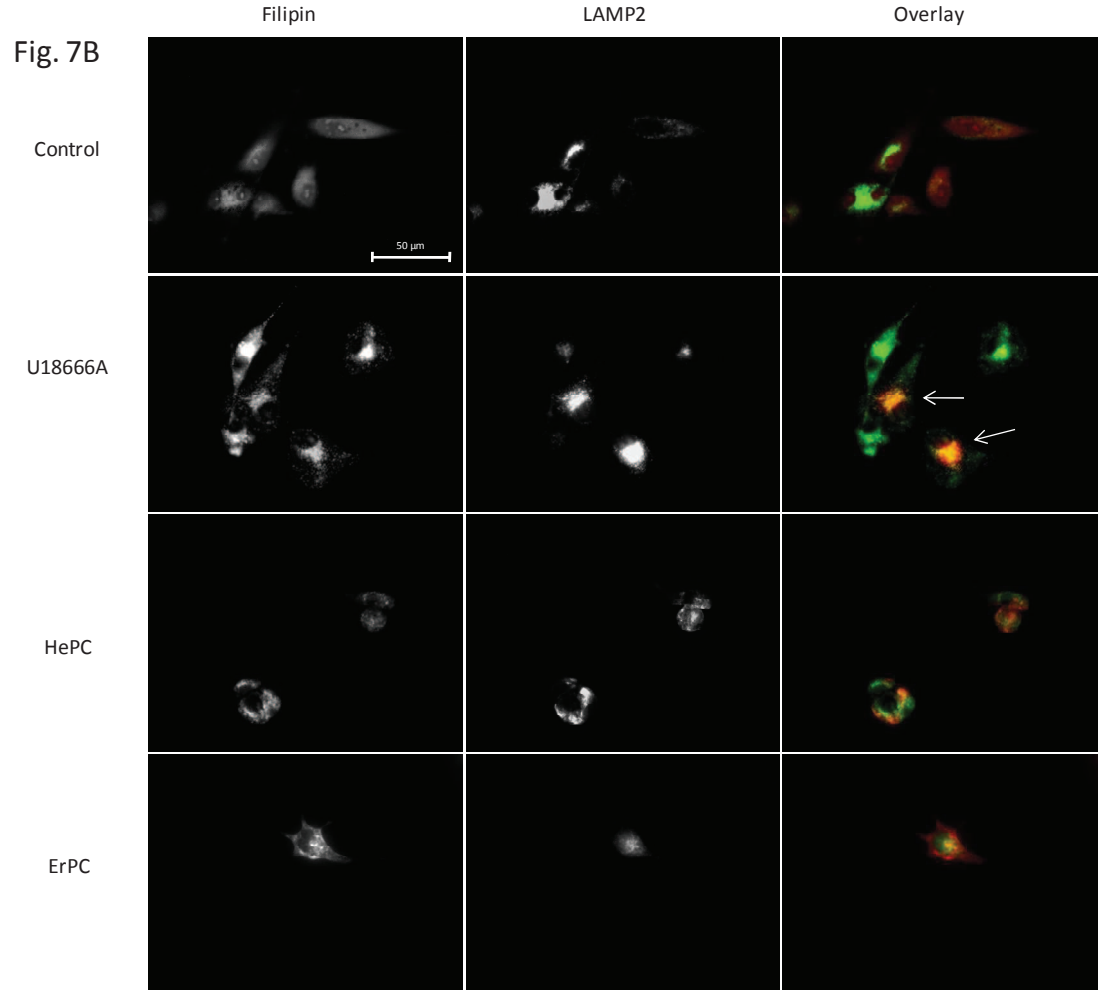


Figure 7. Studies of cholesterol colocalization with endosomal compartments. U-87 MG cells growing in log-phase were treated with vehicle, 1 µM U18666A, 25 µM HePC or 25 µM ErPC for 24 h. They were fixed and washed as described in Materials and methods. Filipin was added for 30 min. The cells were washed with PBS/Tween and samples were incubated overnight with primary antibodies against clathrin heavy chain or LAMP2. Secondary Alexa 488 or 568 antibodies were added for 1 h. In overlay pictures filipin labelling is shown in red and clathrin (A) and LAMP2 (B) are both in green. Overlays show no colocalization between cholesterol and clathrin in any treatment. LAMP2 shows colocalization with filipin in U18666A-treated cells (arrows) but not with APL treatment.

Fig 8.

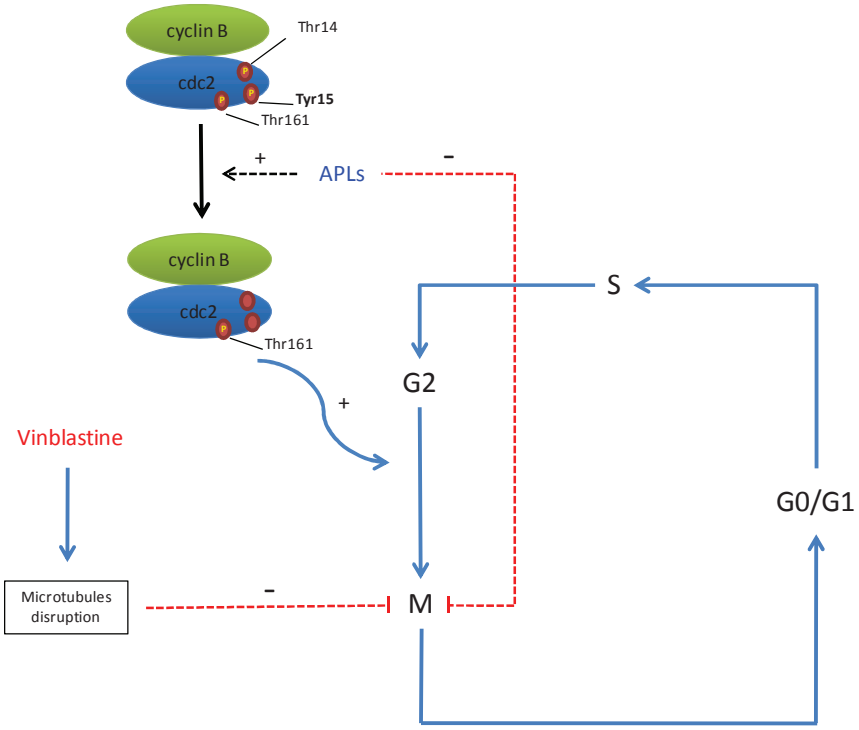


Figure 8. Cell-cycle diagram and APLs. Cell-cycle phases and the G2 to M control point. When complex cyclinB/Cdc2 is active the entrance to mitosis is allowed. Dephosphorylation of residues Thr 14 and Tyr 15 is required for complex activation; when this happens the cell cycle continues to phase M. APLs promote dephosphorylation of residue Tyr 15, which entails not stopping at phase G2. Due to the arrest induced by APLs detected at phase G2/M we postulate that they arrest the cell cycle at some point during the M phase. Vinblastine's mechanism of action is shown.

	Control	HePC	ErPC	Edelfosine	Perifosine
G0/G1	52.50 ± 0.69	54,23 ± 0,70	47,96 ± 2,53	53,99 ± 4,38	40,46 ± 4.55
G2/M	16.19 ± 0.42	17,29 ± 0,56	19,48 ± 1,03*	31,61 ± 2,59**	32,71 ± 2,84**
S	29.27 ± 0.73	28,94 ± 0,79	34,18 ±1.55	20,84 ± 1,71	29,14 ± 6.42

Table 1. Cell-cycle phases and G2/S arrest. The table shows the percentage of U-87 MG cells in the different cell-cycle phases: G0/G1, G2/M and S. The percentage of cells increases during the G2/M phase in the treatments with 25 μM APL or 2.5 mg/ml vinblastine. No significant changes are discernible in the G0/G1 or S phases. This table summarizes the results of three separate experiments. *P < 0.05; **P < 0.005.

4.5. Antitumoral alkylphospholipids induce cholesterol efflux from the plasma membrane in HepG2 cells

The cholesterol homeostasis depends on the balance between uptake, transport, synthesis and efflux from the cell. In the previous works, APLs were shown to alter cholesterol homeostasis by disrupting its transport and synthesis within the cell. As an essential factor in cholesterol homeostasis, in this work we analyse if the cholesterol efflux could be affected by APLs. We decided to run some assays in free serum to avoid the interaction between APL and lipoprotein contained in serum. Interestingly, in this work we report for the first time the capacity of APL to induce the release of plasma membrane cholesterol to the medium.

We performed several assays in HepG2 cells, radiolabeling plasma membrane cholesterol and exposed cells to HePC in free-serum medium. From beginning of the exposition to miltefosine up to 30-45 min, aliquots of medium were taken and a time-course of the radioactivity released to medium was determined. We found out that this agent promote cholesterol sorting from cell in a dose-dependent manner. The efflux kinetic consists on a fast cholesterol release to medium - up to 80% during first 5 min - and a smaller constant rate stabilized around 15-20 min. Cholesterol sorting caused by HePC was markedly more effective than the released by methyl-β cyclodextrin (MβCD), a well know cholesterol depleting agent. Coaddition of cholesterol to different APLs showed that cholesterol efflux was impeded significantly, suggesting an interaction between cholesterol present in medium and APLs. In spite of this, ErPC showed some differences in cholesterol extraction ability compared to the others APLs, as this action was not dependent on its concentration nor affected by cholesterol coadition. Interestingly, inhibition of cholesterol transport to ER was confirmed in cells treated with APL in free-serum solutions, as well as with cholesterol coadded. This indicates that this inhibition, described in the previous works, is unrelated with APLs´ ability to extract cholesterol from plasma membrane to medium.

At this point we checked if cholesterol depletion in HepG2 cells could affect its morphology. Cells were incubated with APLs in free-serum medium with or without cholesterol. All APLs provoked a marked cell rounding in free serum media but clearly not in media supplemented with cholesterol. This indicates that cholesterol extraction alters cell

morphology significantly but if cholesterol depletion is impeded morphology is not affected, at least after short-time treatment.

AKT/PI3K pathway is involved in cell growth, proliferation and survival and, as stated in Introduction, is proposed to be a good target for tumour proliferation. MβCD has been described to cause cell rounding and inhibition of AKT/PI3K pathway. We thence compared MβCD and APLs action on activation status of AKT in HepG2 cells. Interestingly both agents cause cell rounding and inhibit AKT phosphorylation. However, whereas in MβCD-treatment these effects were reverted by cholesterol repletion, in APL-treated cells they were not.

This work provides experimental situations similar to that used in some laboratories when working with APLs in free-serum solutions; other authors should have these results in mind for experiments performed in such conditions. Additionally, cholesterol extraction by APLs has been described for the first time in this work and could explain different cytostatic potencies obtained with APLs in free-serum situations (i.e. lower antiproliferative IC_{50}).

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Antitumoral Alkylphospholipids Induce Cholesterol Efflux from the Plasma Membrane in HepG2 Cells

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ABSTRACT

Alkylphospholipid (APL) analogs are promising candidates in the search for treatments of cancer. Previous studies conducted in our laboratory indicate that, after prolonged treatment, they alter cholesterol homeostasis in HepG2 cells. Here we describe the effects that different APLs exert upon this cell line after a 1-h exposure in a serum-free medium, including 1) a rapid, significant increase in cholesterol efflux into the extracellular medium, which consequently provoked a depletion of cholesterol in the plasma membrane (further assays conducted in an attempt to return to control cholesterol levels were only partially successful); 2) use of methyl-β-cyclodextrin, which indicated that APLs acted in a way similar to this agent that is used frequently to modulate membrane cholesterol levels; 3) the phosphorylation of Akt that showed that this critical regulator for cell survival was modulated by changes in cholesterol levels induced in the plasma membrane by APLs; and 4) membrane cholesterol depletion that is not related to the impairment of cholesterol traffic produced by APLs. Thus, we have found that antitumoral APLs efficiently deplete membrane cholesterol, which may be one important factor in determining the early biological actions of APLs.

Introduction

Alkylphospholipid (APL) analogs are a new class of antitumoral agents that do not target DNA but insert themselves into the plasma membrane and subsequently trigger a broad range of biological effects, which ultimately lead to cell death (Soto and Soto, 2006; Rakotomanga et al., 2007). An important characteristic of APLs is their amphiphilic properties, enabling them to interact with cell membranes and affect cell metabolism at different levels (reviewed by Jiménez-López et al., 2010). Within this context, studies carried out in our laboratory have demonstrated that, after long-term treatment, the APL hexadecyl 2-(trimethylazaniumyl)ethyl phosphate (hexadecylphosphocholine; HePC), also known as miltefosine, alters phosphatidylcholine metabolism (Jiménez-López et al., 2004) and intracellular cholesterol trafficking and metabolism, all of which lead to an increased uptake, synthesis, and accumulation of cholesterol in the cell (Jiménez-López et al., 2006; Carrasco et al., 2008; Marco et al., 2009). Thus, we extended our studies to analyze the effects of a variety of APLs, such as [1-*O*-octadecyl-2-*O*-methyl-rac-glycero-3-phosphocholine] (edelfosine), [(*Z*)-docos-13-enyl] 2-(trimethylazaniumyl)ethyl phosphate (erucylphosphocholine; ErPC), and 1,1-dimethylpiperidin-1-ium-4-yl) octadecyl phosphate (perifosine) upon intracellular cholesterol homeostasis and found that all of them impede the esterification of plasma-membrane cholesterol via acyl-CoA/cholesterol acyltransferase activity (Carrasco et al., 2010). This effect is caused by the disruption of cholesterol movement from the plasma membrane to the endoplasmic reticulum (ER), where it is esterified, which in turn induces a significant cholesterogenic response in HepG2 cells, involving increased gene expression and higher levels of several proteins involved in the pathway for the biosynthesis and receptor-mediated uptake of cholesterol.

Cell-cholesterol levels are the result of a balance among uptake, efflux, and endogenous synthesis. Many aspects of cholesterol metabolism are well known, including its uptake, its synthesis in the ER, and its regulation via sterol regulatory element-binding protein. Nevertheless, although widely investigated, some dispute remains on how cholesterol mol-

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doi:10.1124/jpet.110.172890.

ABBREVIATIONS: APL, alkylphospholipid; ER, endoplasmic reticulum; ErPC, [(*Z*)-docos-13-enyl] 2-(trimethylazaniumyl)ethyl phosphate (erucylphosphocholine); HePC, hexadecyl 2-(trimethylazaniumyl)ethyl phosphate (hexadecylphosphocholine); MβCD, methyl-β-cyclodextrin; PBS, phosphate-buffered saline; BSA, bovine serum albumin; HRP, horseradish peroxidase; MEM, minimal essential medium; FBS, fetal bovine serum; IGF, insulin-like growth factor.

ecules move from the plasma membrane to extracellular acceptors. The generally recognized mechanism is now thought to involve the movement of cholesterol molecules from the cell membrane through the aqueous phase to the acceptor particle, that is to say, by an aqueous diffusion mechanism. Other mechanisms have also been proposed, but with the inclusion of collision or receptor mediation (Fielding and Fielding, 2001).

The concentration of cholesterol in the membrane is particularly high in lipid rafts, which have been reported to act as molecular platforms and play a significant role in many signaling cascades (Simons and Toomre, 2000) and in the activation of immune responses (Langlet et al., 2000). Recent studies with cultured cells have suggested that APLs may act on cell signal transduction by affecting the protein composition of these rafts. Thus, treatment of human leukemia cells with edelfosine results in the translocation of Fas into membrane rafts, and this apparently triggers apoptosis (Mollinedo et al., 2010). Another raft-dependent pathway shown to be an important target of APL is the phosphatidylinositol 3-kinase/Akt pathway, which is involved in cell growth, proliferation, and survival (Engel et al., 2008).

In the present work, we have extended our studies to analyze the effects of clinically relevant APLs upon cholesterol efflux in the human hepatoblastoma cell line HepG2, which is commonly used for lipid metabolism studies because it retains many liver-specific functions. Our objective was to analyze the initial effects of APLs upon cholesterol efflux into the medium, one of the most important processes contributing to the control of cell cholesterol levels in the hope that this would provide us with information about the early mechanism of APL activity on cholesterol homeostasis. We found that all of the APLs assayed, HePC, edelfosine, ErPC, and perifosine, stimulated the efflux of cholesterol from cells into the medium but that this could be mitigated by the addition of exogenous cholesterol. The cholesterol release provoked a depletion of cholesterol levels in the plasma membrane that can be related to the activation state of Akt.

Materials and Methods

Chemicals and Reagents. Fetal bovine serum (FBS) was from The Cell Culture Company (Pasching, Austria). Minimal essential medium (MEM), cholesterol, water-soluble cholesterol, methyl-β-cyclodextrin (MβCD), and thin layer chromatography plates were from Sigma-Aldrich (Madrid, Spain). X-ray film was from Konica Minolta (Tokyo, Japan). [7(n)$^{3-3}$H]cholesterol was from PerkinElmer Life and Analytical Sciences (Waltham, MA). HePC was from Cayman Chemical (Ann Arbor, MI), edelfosine was from Calbiochem (Nottingham, UK), ErPC was from Alexis Biochemicals (Exeter, UK), and perifosine was from Selleck Chemicals (London, ON, Canada). Polyclonal anti-human phospho-Akt (Ser473) antibody and horseradish peroxidase (HRP)-linked secondary IgG were from Cell Signaling Technology (Danvers, MA). Polyclonal anti-human Akt1/2/3 primary antibody was from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). The enhanced chemiluminescence (ECL) detection system was from Millipore (Billerica, MA).

Cell Culture. The human hepatoblastoma HepG2 cell line was from The European Collection of Animal Cell Cultures (Salisbury, UK). Cells were cultured in MEM containing 10% heat-inactivated FBS supplemented with 2 mM L-glutamine, 1% nonessential amino acids, 100 U/ml penicillin, and 100 μg/ml streptomycin. Cells were grown in a humid atmosphere with 5% CO_2 at 37°C and subcultured at a ratio of 1:10 once a week. Cells were plated on tissue-culture dishes (Nalge Nunc International, Rochester, NY) at a density of 5×10^4 cells/cm^2 and kept in culture medium before use in experimental assays at approximately 70% confluence.

Cholesterol Efflux Assays. HepG2 cells were cultured in MEM/10% FBS for 24 h. The medium was removed, and the cells were washed twice with PBS. To label the plasma membrane, radioactive [7(n)^3-^{3}H]cholesterol (2 μCi/ml) was added in a serum-free medium for a period of 60 min. To remove any unincorporated radioactivity, the cells were washed three times with PBS containing 0.5% BSA and twice with PBS (both prewarmed to 37°C). The different treatments were added in serum-free medium, and aliquots were collected at various intervals from 0 to 45 min. Radiometric measurements of medium aliquots were made by liquid scintillation using a Beckman 6000-TA counter (Beckman Coulter, Madrid, Spain) and expressed in terms of percentage as radioactive cholesterol in medium per total radioactive cholesterol. Background (control) values were subtracted from treatment values.

Trafficking of Cholesterol from the Plasma Membrane to the Endoplasmic Reticulum. An efficient way of measuring cholesterol transport from the plasma membrane to the ER is to determine the degree of esterification of radiolabeled cholesterol previously incorporated into the plasma membrane (Lange and Steck, 1997; Marco et al., 2009). Thus, HepG2 cells were incubated with 2 μCi/ml [7(n)^3-^{3}H]cholesterol for 60 min at 37°C and then with the different APLs in the presence or absence of 30 μg/ml cholesterol. After 45 min of exposure, the medium was removed, and the lipids were extracted from the cells following the procedure of Bligh and Dyer (1959). Cholesterol and cholesteryl esters were separated by thin layer chromatography using a mixture of hexane/diethyl ether/acetic acid (70:30:2) as solvent. Radiometric measurements of scraped lipid spots, rendered visible by exposure to iodine vapor, were made by liquid scintillation. The fraction of esterified plasma-membrane cholesterol is expressed in terms of the percentage of esterification of the total labeled cholesterol.

Study of Cholesterol Replenishment. HepG2 cells were cultured in MEM/10% FBS for 24 h. The medium was removed, and cells were washed twice with PBS before being treated with 50 μM edelfosine or 5 mM MβCD (as positive control) or with no additions (control) for 60 min. To quantify cholesterol, lipids were extracted from half of the replicates, as described above. The other half of the samples was washed extensively with PBS to remove edelfosine or MβCD, and 1 mM water-soluble cholesterol in MEM was added. After 90 min, the cells were visualized under an inverted microscope, and lipid extraction and chromatography were carried out. Cholesterol levels were determined with the use of an enzymatic colorimetric kit from Lab Kit (Madrid, Spain).

Immunoblot Analysis of Akt Activation. HepG2 cells growing in log phase were deprived of serum overnight and incubated with MEM in the absence (only vehicle) or presence of 20 μM edelfosine, 20 μM perifosine, or 5 mM MβCD for 30 min. To stimulate Akt, the cells were incubated with 100 nM human insulin-like growth factor (IGF)-1 for 15 min, and the phosphorylation state of Akt was measured. In addition, the effect of cholesterol replenishment on basal Akt activation was analyzed after washing three times with PBS supplemented with 0.5% fatty acid-free BSA and further incubation with 1 mM water-soluble cholesterol for 45 min. After treatment, the cells were washed twice, scraped into ice-cold PBS, pH 7.4, and centrifuged at 100g for 10 min at 4°C. Cell pellets were suspended in 0.1 ml of ice-cold lysis buffer consisting of 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% Triton X-100, and a protease inhibitor cocktail from Sigma-Aldrich and incubated on ice for 30 min with occasional shaking. Cell lysates were centrifuged at 10,000g for 15 min at 4°C, and the supernatants were stored at −80°C until use; an aliquot was taken to determine protein concentration. Equal amounts of lysate protein (60 μg) were separated by 10% SDS-polyacrylamide gel electrophoresis and transferred onto polyvinylidene difluoride membranes. Prestained broad-range protein-molecule mass markers were used during electrophoresis. Membranes were blocked in PBS

containing 5% nonfat dry milk and 0.1% Tween 20 and then incubated with phospho-Akt primary antibody (1:1000) in blocking solution at 4°C with gentle shaking overnight. After several washes in PBS containing 0.1% Tween 20, the membranes were incubated with HRP-conjugated IgG (1:5000) as secondary antibody for 60 min. Immunoreactive proteins were detected by autoradiography using a chemiluminescent HRP substrate and exposure to X-ray film. After incubation with an antibody-stripping solution consisting of 60 mM Tris-HCl, pH 6.8, 100 mM β-mercaptoethanol, and 2% SDS for 30 min at 60°C, blots were incubated with total-Akt primary antibody (1:1000). Densitometric analysis was carried out using the ImageJ gel-digitizing software (http://rsbweb.nih.gov/ij/).

Statistics. Results are expressed as mean ± S.E.M. One-way analysis of variance with post hoc comparisons by Scheffé's test was carried out (SPSS 13.0; SPSS, Inc., Chicago, IL). P values <0.05 were considered to be statistically significant.

Other Analyses. Cell-protein content was determined in the cell homogenates by the Bradford (1976) method using BSA as standard. Cell morphology was observed under an inverted microscope.

Results

Effects of Hexadecylphosphocholine on Cholesterol Efflux. We recently reported that long-term exposure to HePC causes deregulation in the homeostasis of cholesterol in HepG2 cells, both in terms of synthesis and intake, but we had not yet explored the possible effects of HePC on cholesterol efflux. Therefore, in this work, we have extended our studies to analyze further the possible manner in which HePC might influence cholesterol efflux. To this end, we treated cells with [7(n)^3-^{3}H]cholesterol for 60 min to label the plasma membrane and then exposed them to different quantities of HePC, determining the recovery of the [7(n)^3-^{3}H]cholesterol in the medium after a 45-min incubation. Because serum acts as an extracellular cholesterol acceptor, the studies on cholesterol efflux were routinely conducted in a serum-free medium. As can be seen in Fig. 1, HePC significantly increases cholesterol efflux from the cells in a dose-dependent manner. Thus, in the presence of 50 μM HePC, HepG2 cells released up to 40% of total radiolabeled plasma-membrane cholesterol. Bearing these results in mind, we determined the time course of cholesterol efflux in cells exposed to 50 μM HePC (the most efficient concentration assayed) and compared it with cholesterol efflux in cells exposed to MβCD, a well known cholesterol-depleting agent. We then labeled the cholesterol in plasma membrane for 60 min and followed the kinetics of cholesterol efflux both in the presence of HePC or MβCD and in their absence as controls. As has been shown in other cell types (Zidovetzki and Levitan, 2007), when HepG2 cells were exposed to 5 mM MβCD, we observed the existence of two distinct plasma membrane cholesterol pools, a fast pool removed with a half-time ($t_{1/2}$) of 85 s and a slow pool with a $t_{1/2}$ of 10 min. Although at 30 min, 5 mM MβCD was significantly more efficient in releasing cholesterol than 50 μM HePC (*, $P < 0.001$), the efflux kinetics were similar (Fig. 2). It is noteworthy that the cholesterol efflux induced at 30 min by 50 μM HePC was significantly higher (*, $P < 0.001$) than that observed in cells treated with the same concentration of MβCD.

Alkylphospholipid-Dependent Efflux Modulation by Cholesterol Coaddition. The results shown in Figs. 1 and 2 prompted us to see whether the action of HePC on the efflux of cholesterol could be affected by the presence of exogenous cholesterol. Thus, we assayed the rate of plasma-membrane [7(n)^3-^{3}H]cholesterol efflux in the presence of 50 μM HePC and different quantities of cholesterol and found that the addition of 15 μg/ml cholesterol clearly reduced the quantity of cholesterol released into the medium without affecting the efflux kinetics (Fig. 3). It is noteworthy that the levels of cholesterol higher than 30 μg/ml abrogated the efflux of cholesterol induced by HePC.

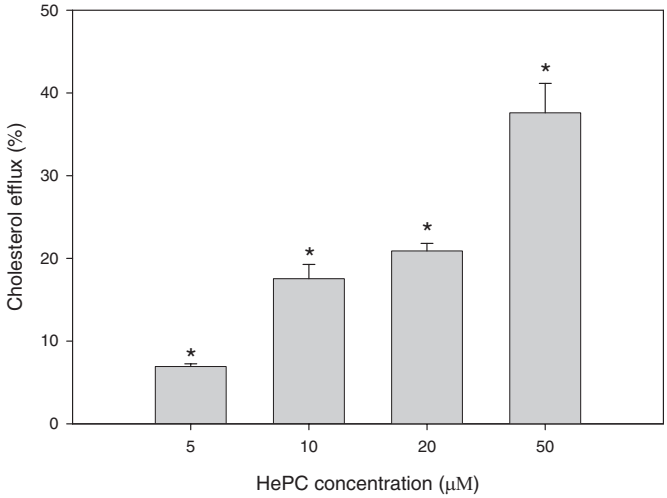

Fig. 1. Effect of hexadecylphosphocholine on cholesterol efflux. Log-phase HepG2 cells grown for 24 h in MEM containing 10% FBS. Cells were washed with PBS, and then 2 μCi/ml [7(n)^3-^{3}H]cholesterol was added in serum-free MEM within 60 min. They were then washed with PBS containing 0.5% albumin and PBS at 37°C and subsequently treated without any additions (control) or with 5, 10, 20, and 50 μM HePC for 45 min. Radioactive cholesterol was measured as described under *Materials and Methods*. Cholesterol efflux is expressed in terms of percentage as radioactive cholesterol in medium per total radioactive cholesterol and represents the mean ± S.E.M. of three independent experiments conducted in triplicate (*, $P < 0.05$ compared with control).

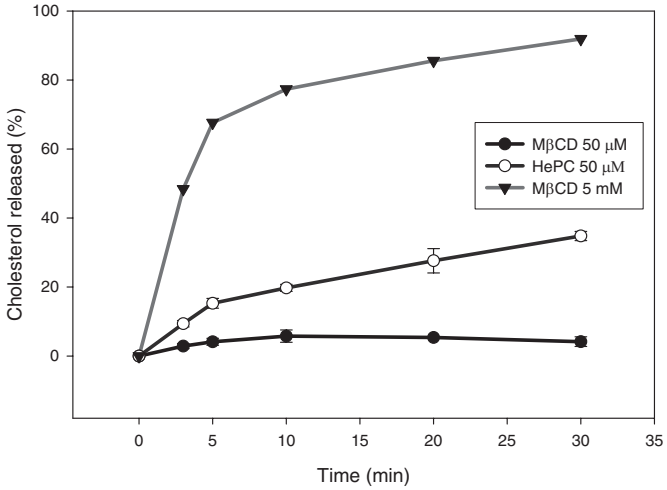

Fig. 2. Cholesterol efflux kinetics. Log-phase HepG2 cells grown for 24 h in MEM containing 10% FBS. Cells were washed with PBS, and then 2 μCi/ml [7(n)^3-^{3}H]cholesterol was added in serum-free MEM within 60 min. They were then washed with PBS containing 0.5% albumin and PBS at 37°C and subsequently treated with 50 μM HePC, 50 μM MβCD, or 5 mM MβCD for 30 min. Aliquots of medium were collected after 0, 3, 5, 10, 20, and 30 min. Radioactive cholesterol was measured as described under *Materials and Methods*. Cholesterol efflux is expressed in terms of percentage as radioactive cholesterol in medium per total radioactive cholesterol and represents the mean ± S.E.M. of two independent experiments conducted in triplicate.

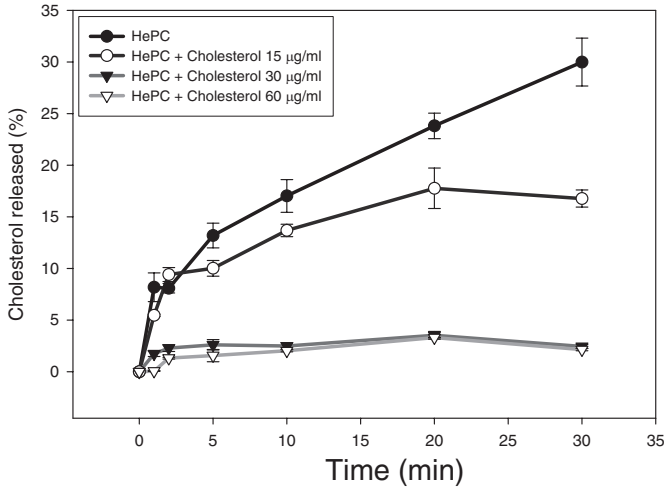

Fig. 3. Effect of cholesterol coaddition on efflux kinetics. Log-phase HepG2 cells grown for 24 h in MEM containing 10% FBS. Cells were washed with PBS, and then 2 μCi/ml [7(*n*)^3-^{3}H]cholesterol was added in serum-free MEM within 60 min. They were washed with PBS containing 0.5% albumin and PBS at 37°C and then treated with only 50 μM HePC or 50 μM HePC plus 15, 30, or 60 μg/ml cholesterol for 30 min. Aliquots of medium were collected after 0, 1, 2, 5, 10, 20, and 30 min. Radioactive cholesterol was measured as described under *Materials and Methods*. Cholesterol efflux is expressed in terms of percentage as radioactive cholesterol in medium per total radioactive cholesterol and represents the mean ± S.E.M. of two independent experiments conducted in triplicate.

Cholesterol Efflux Induced by Other Alkylphospholipid Analogs. Because we recently demonstrated that other APLs, such as edelfosine, ErPC, and perifosine, disrupt cholesterol homeostasis in HepG2 cells via a mechanism similar to that of HePC (Carrasco et al., 2010), we went on to study the influence of these APLs on cholesterol efflux both in the presence and absence of exogenous cholesterol. The high capacity of the APLs to stimulate cholesterol efflux is shown in Table 1. As can be seen, three of the APLs assayed induced a concentration-dependent release of cholesterol, with edelfosine clearly being the most efficient since nearly 50% of plasma-membrane cholesterol from the HepG2 cells found in the medium after 30 min of incubation. Cholesterol efflux in the presence of the four APLs followed the pattern: edelfosine > HePC > perifosine > ErPC. It is noteworthy that ErPC exhibited a clear difference in its ability to release cholesterol from the cell given that all of the concentrations of this APL assayed resulted in effluxes of similar quantities of cholesterol into the medium. What was noticeable, however, was that all of the APLs analyzed exhibited similar efflux kinetics to that observed with HePC (data not shown). As far as the coaddition of cholesterol is concerned, in just the same way as we had observed previously with HePC, we found that exogenous cholesterol reduced the efficacy of edelfosine and perifosine to stimulate cholesterol efflux, whereas it had little effect upon ErPC.

TABLE 1

Measurement of cholesterol efflux caused by alkylphospholipids: cholesterol coaddition

Log-phase HepG2 cells grown for 24 h in MEM containing 10% FBS. Cells were washed with PBS and then 2 μCi/ml [7(*n*)^3-^{3}H]cholesterol was added in serum-free MEM within 60 min. They were then washed with PBS containing 0.5% albumin, and PBS at 37°C and then treated with or without 10, 20, and 50 μM HePC, edelfosine, ErPC, or perifosine in the absence or presence of 30 μg/ml cholesterol for 30 min. Radioactive cholesterol was measured as described under *Materials and Methods*. Cholesterol efflux is expressed in terms of percentage as radioactive cholesterol in medium per total radioactive cholesterol and represents the mean ± S.E.M. of three independent experiments conducted in triplicate.

	Chol −	Chol +
HePC		
10 μM	14.0 ± 0.8*	0.5 ± 0.1**
20 μM	16.7 ± 0.5*	5.4 ± 1.1**
50 μM	29.8 ± 2.1*	10.9 ± 2.1**
Edelfosine		
10 μM	4.5 ± 0.6	2.4 ± 0.1
20 μM	30.1 ± 1.7*	1.1 ± 0.1**
50 μM	49.3 ± 2.4*	3.0 ± 0.2**
ErPC		
10 μM	10.8 ± 2.0	14.9 ± 1.6
20 μM	9.1 ± 0.6*	12.8 ± 2.4
50 μM	11.5 ± 2.2	11.9 ± 2.1
Perifosine		
10 μM	5.7 ± 0.6*	0.9 ± 0.1**
20 μM	12.8 ± 1.7*	5.1 ± 0.1**
50 μM	17.4 ± 0.9*	8.4 ± 0.7**

* $P < 0.05$ compared to control.
** $P < 0.05$ compared with APL without cholesterol coaddition.

Cholesterol Coaddition Does Not Impede the Interruption of Cholesterol Traffic Induced by APLs. We also studied the manner in which APLs interfered in the traffic of cholesterol from the plasma membrane to the ER under conditions of enhanced cholesterol efflux (i.e., after exposure to only APL) and after exposure with APL plus cholesterol, where cholesterol efflux is not modified by the APL. Considering that the best experimental procedure to study any interference in the traffic of cholesterol from the plasma membrane to the ER is to analyze the rate of synthesis of esterified cholesterol from plasma-membrane cholesterol, we labeled the cholesterol in the plasma membrane and then exposed the HepG2 cells to the different APLs, both in the presence and absence of 30 μg/ml exogenous cholesterol, and determined the radioactivity appearing in the esterified cholesterol. The data in Fig. 4 show clearly that, as we have reported in a previous publication (Carrasco et al., 2010), all of the APLs assayed impeded the arrival of plasma-membrane

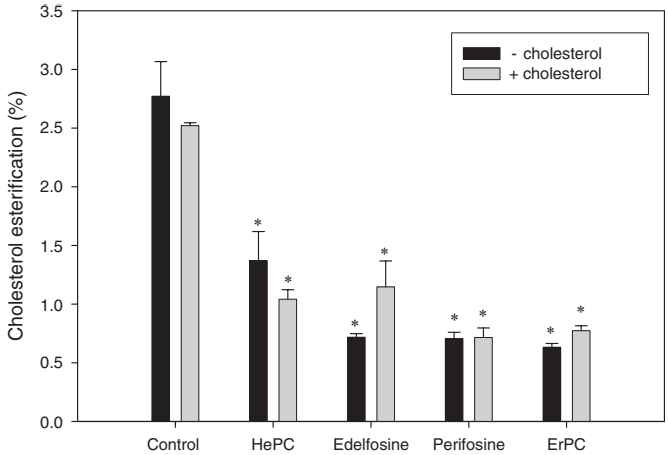

Fig. 4. Effect of alkylphospholipids and cholesterol coaddition on cholesterol traffic. Log-phase HepG2 cells were seeded and allowed to grow for 24 h in MEM containing 10% FBS. Cells were washed with PBS, and then 2 μCi/ml [7(*n*)^3-^{3}H]cholesterol was added in serum-free MEM within 60 min. They were then washed with PBS containing 0.5% albumin and PBS at 37°C and subsequently treated without any additions (control) or with 50 μM HePC, 20 μM edelfosine, 50 μM ErPC, or 20 μM perifosine in the absence or presence of 30 μg/ml cholesterol for 30 min. Radioactive cholesterol was measured as described under *Materials and Methods*. The fraction of plasma-membrane cholesterol that was esterified is expressed in terms of the percentage of esterification of the total labeled cholesterol and represents the mean ± S.E.M. of three independent experiments conducted in triplicate (*, $P < 0.05$ compared with the corresponding control).

cholesterol to the ER and thus its subsequent esterification but also that this interference was unrelated to the release of plasma-membrane cholesterol because it was observed both in the presence and absence of exogenous cholesterol.

Effects of Alkylphospholipids on Cell Morphology. Cells exposed to the different APLs for 30 min became abnormally rounded in shape. A similar morphological change has been reported by other authors in cells depleted of cholesterol by exposure to MβCD (Park et al., 2009). Thus, in our study, we further examined whether the presence of cholesterol might prevent the changes in cell morphology induced by APLs. As illustrated in Fig. 5, HePC and edelfosine treatments clearly brought about changes in cell morphology, from flattened to rounded within 30 min. It is interesting to note that according to the cholesterol studies reported above (Table 1), this change was broadly mitigated by the coaddition of cholesterol. We observed the same effects with the other APLs (data not shown).

Study of Cholesterol Replenishment in Alkylphospholipid-Treated Cells. We have demonstrated in this study that exposure of HepG2 cells to APLs stimulates cholesterol efflux from the cell surface and also causes the cells to become round in shape. It has also been reported recently that cholesterol depletion produced by either MβCD or statins induces cell death concomitantly with a decrease in levels of cell-surface lipid rafts (Li et al., 2006), which are restored by cholesterol replenishment. Thus, we went on to examine whether stimulation of the efflux of cholesterol induced by APLs was able to deplete cholesterol levels in the HepG2 cells and, if this were so, whether subsequent incubation with exogenous cholesterol could replenish these cholesterol levels and restore normal cell morphology. To this end, we chose edelfosine as the representative APL. After a 60-min exposure to edelfosine or MβCD (as positive control) and further washings with PBS, we added 1 mM water-soluble cholesterol. In accordance with previous reports by several authors working with other cell lines (Haynes et al., 2000; Rouquette-Jazdanian et al., 2006), we found that approximately 60% cellular cholesterol was depleted by MβCD from the HepG2 cells (Fig. 6). It is noteworthy that 50 μM edelfosine also stimulated the efflux of similar quantities of cholesterol after the same time. Apart from this, both cholesterol levels and the morphology of the MβCD-treated cells returned to the control situation after cholesterol replenishment, whereas edelfosine-treated cells showed no improvement in morphology or a complete return to control cholesterol levels.

Comparative Analysis of the Effects of Methyl-β-cyclodextrin and Alkylphospholipids on the Activation Status of Akt. To study the possible role of APLs on Akt activity, we conducted immunoblotting assays to analyze the effect caused by edelfosine and perifosine on the level of phosphorylated active Akt after stimulation with IGF-1. Exposure of the cells to edelfosine, perifosine, or 5 mM MβCD for 30 min produced a significant decrease in the phospho-Akt/Akt ratio in the assayed cell lysates, as a result of reduced phospho-Akt, after IGF-1 stimulation (Fig. 7A). Figure 7B shows that cholesterol replenishment restores the state of phosphorylation of Akt in MβCD-treated cells but not in the APL-exposed cells.

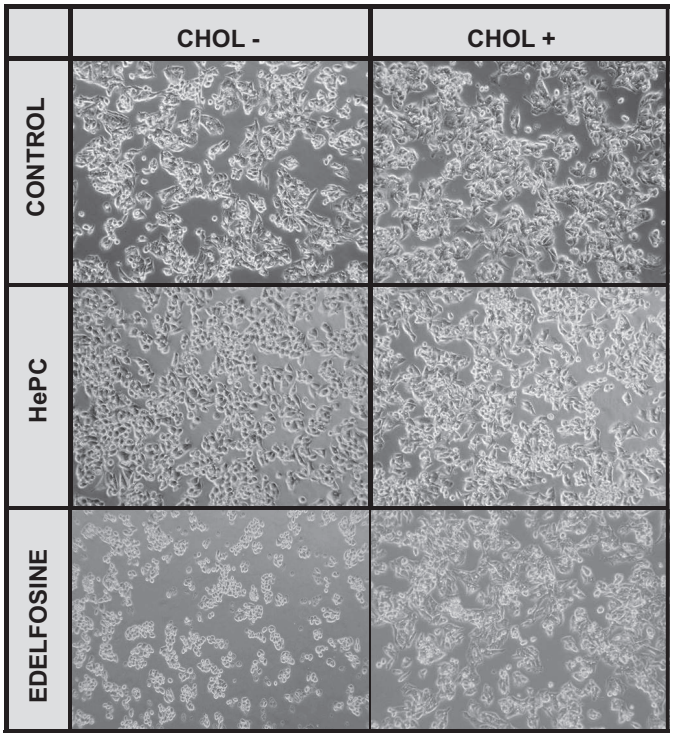

Fig. 5. Morphological changes induced by alkylphospholipids/cholesterol coaddition. Log-phase HepG2 cells grown for 24 h in MEM containing 10% FBS. The cells were washed with PBS and then incubated in serum-free MEM without any additions (control) or with 50 μM HePC or 50 μM edelfosine—all of them with or without 30 μg/ml cholesterol for 30 min. The cells were examined and photographed with an inverted microscope (20× magnification).

Discussion

Our laboratory has reported that HePC alters cholesterol homeostasis in HepG2 cells by impairing cholesterol trafficking from the plasma membrane to the ER (Marco et al., 2009). More recently, we have extended our studies and demonstrated that other APLs, such as edelfosine, perifosine, and ErPC, produce similar effects on cholesterol homeostasis, demonstrating that all of the APLs assayed exhibited a common mechanism of action (Carrasco et al., 2010). In this work, we analyze the early action mechanism of these APLs, which may trigger the alterations in cholesterol homeostasis produced by these compounds. Because intracellular cholesterol metabolism is controlled by influx, synthesis, and efflux, we investigated the possible effect of APLs on the release of cellular cholesterol into a serum-free medium (i.e., in the absence of any physiological or synthetic acceptors). Our data demonstrate that exposure to HePC (as well as edelfosine, perifosine, and ErPC) results in a significant efflux of cholesterol from the plasma membrane into the extracellular medium in HepG2 cells. This effect has not been described until now and is of great interest because in vitro biophysical and biochemical studies into APL activity are frequently carried out in a serum-free medium where cholesterol is released by APLs. This should be born in mind when interpreting some of the actions of APLs.

Several studies have shown that APLs are surface-active molecules, with a high affinity for membranes but weak detergent activity (Busto et al., 2007). Because of their inverted-cone shape, HePC, edelfosine, and perifosine disperse in water in the form of micelles (Busto et al., 2008), display-

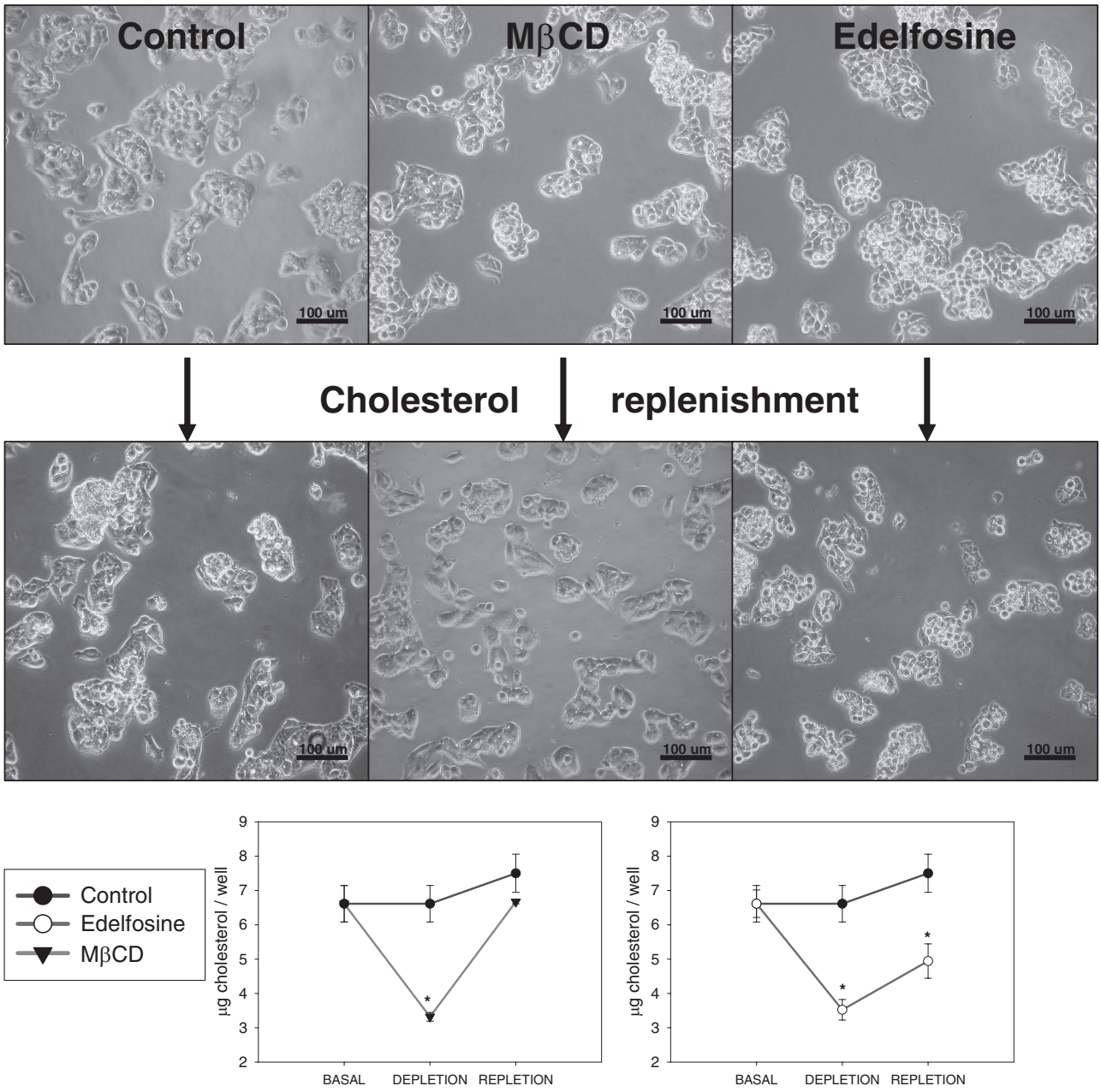

Fig. 6. Cholesterol replenishment in cells treated with alkylphospholipids. Log-phase HepG2 cells were grown for 24 h in MEM containing 10% FBS. The cells were washed with PBS and then incubated in serum-free MEM without any additions (control) or with 50 μM edelfosine or 5 mM MβCD for 60 min. The cells visualized under an inverted microscope (20× magnification) were photographed, and cholesterol levels were measured. After being washed with PBS, the cells were incubated with only MEM containing 1 mM water-soluble cholesterol for 90 min. Cells were photographed again, and cholesterol levels were measured. Levels are represented as micrograms per cholesterol of sample in three states: basal, depletion (t = 60 min), and replenishment (t = 150 min) (*, $P < 0.05$ compared with control).

ing a critical micellar concentration at 2.5 to 3 μM (Rakotomanga et al., 2004). As mentioned under *Results*, ErPC exhibits a behavior different from that of other APLs on cholesterol release from the plasma membrane. This may possibly be attributed to the fact that ErPC, because of its unsaturated *cis*-13-docosenol derivative structure, forms lamellar structures rather than micelles (Dymond et al., 2008). In any case, as stated above, the efflux of cholesterol into the acceptor-free medium increases significantly in the presence of the different APLs assayed, and we hypothesize that, because of their high affinity for cholesterol, aggregates of APLs may act by removing cholesterol from the cell surface, as do other physiological and synthetic acceptors, such as serum, cyclodextrins, high-density lipoproteins, and phospholipid vesicles.

A comparison between the efficiency of HePC and MβCD for releasing cholesterol reveals that, in 30 min, a 100-fold lower concentration of HePC released around 30% of the cholesterol released by MβCD and that the rates of APL-induced cholesterol efflux were far in excess of those achieved with the same low concentration of MβCD. An analysis of the kinetics of cholesterol efflux in erythrocytes has demonstrated the existence of a fast kinetic pool (Steck et al., 2002), but other studies undertaken with different cell lines have

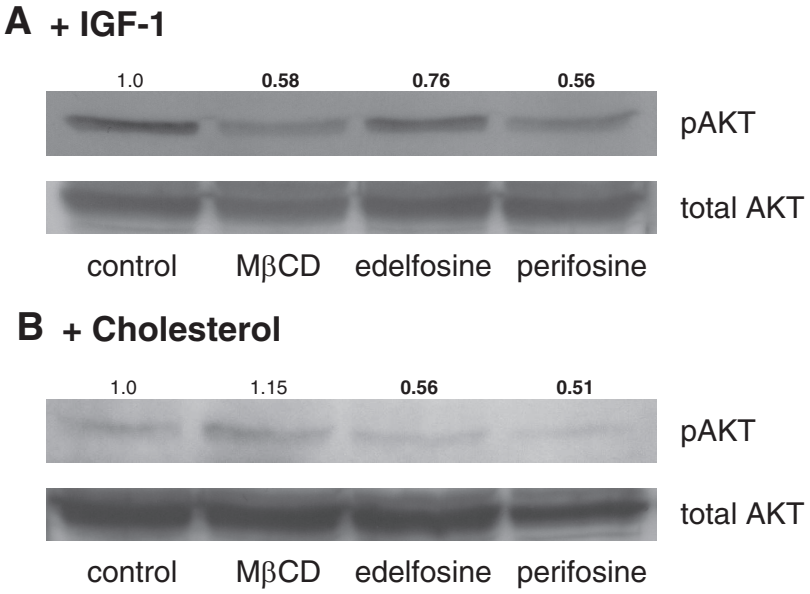

Fig. 7. Effects of methyl-β-cyclodextrin, edelfosine, and perifosine on the activation of Akt. Serum-deprived HepG2 cells were incubated with MEM without any additions (control) or containing 20 μM edelfosine, 20 μM perifosine, or 5 mM MβCD for 30 min. After Akt stimulation with IGF-1, the Akt phosphorylation was measured (A). The effect of cholesterol replenishment on basal Akt activation was also analyzed (B). Cell lysate samples were collected and analyzed by immunoblotting to determine the content of phospho-Akt and total Akt, as described under *Materials and Methods*. The bands were scanned, and arbitrary units were assigned by densitometric analysis. Ratios between the intensity of the bands corresponding to phospho-Akt and Akt are shown and expressed as fold change compared with the corresponding control ratio (1.0).

reported that free cellular cholesterol exists in two kinetic pools (Zidovetzki and Levitan, 2007). Rouquette-Jazdanian et al. (2006) suggest that the fast pool was associated with the raft domains, whereas the slow pool was associated with non–raft-membrane fractions. Our time course efflux experiments also demonstrated that, in HepG2 cells, there were two distinct plasma-membrane cholesterol pools given that the kinetics of cholesterol efflux to 5 mM MβCD was biexponential. It is noteworthy that the efflux kinetics with HePC and other APLs was similar, with a fast cholesterol release lasting a few minutes followed by a slower efflux thereafter.

Of special interest in our study is the fact that, with the exception of ErPC, the coaddition of small quantities of cholesterol (from 15 to 60 μg/ml) with the APLs significantly reduced their capacity to induce cholesterol efflux from the plasma membrane. A similar reduction in the cholesterol-releasing efficiency of MβCD caused by the presence of cholesterol has been reported previously (Li et al., 2006; Park et al., 2009), and it is now accepted, in fact, that MβCD can act as a cholesterol donor-acceptor system because the ratio between the quantities of cholesterol and cyclodextrin in the complexes determines whether it will act as cholesterol acceptor or donor. At the moment, we cannot offer any unequivocal explanation for the cholesterol buffering effect on the APL-induced cholesterol efflux, but it is probable that the capability of APL micelles to accept cholesterol decreases concomitantly with an increase in the quantity of exogenous cholesterol complexed with them. On the other hand, Rakotomanga et al. (2004) have shown that HePC molecules are inserted into the monolayers of lipids as monomers until critical micellar concentration. At higher concentrations, however, HePC micelles are deployed at the interface as groups of monomers in sterol monolayers and so the presence of cholesterol might stabilize the micellar structure, thus decreasing the number of APL molecules that can deabsorb from the micelle to interact with the membrane. This could be the explanation for the well known fact that the effect of APLs is reduced by the presence of serum and other compounds (Ménez et al., 2007), because the interaction of APLs with cholesterol would decrease their effective concentration. Moreover, the reduction in the capability of APLs to release the cholesterol from cells in the presence of exogenous cholesterol may be the reason for the lower hemolytic effect found by other authors in the presence of combinations of APLs and cholesterol (Busto et al., 2008).

It is well documented that the efflux of cholesterol from the plasma membrane to an acceptor molecule in the medium results in the stimulation of 3-hydroxy-3-methylglutaryl-CoA reductase activity and inhibition of cholesterol esterification (Field et al., 1998). Therefore, we wondered whether the possible alteration of plasma-membrane cholesterol caused by the efflux induced by APLs might be involved in the alterations produced by APLs on cholesterol homeostasis reported previously by our research group (Jiménez-López et al., 2010). In this work, we have demonstrated that the interference of APLs on cholesterol traffic from the plasma membrane to the ER is not related to cholesterol efflux, because it is to be seen both when cholesterol is released into the extracellular medium (incubation with APLs alone) and when cholesterol is not released into the medium (cholesterol coadded with APLs).

Furthermore, we went on to analyze whether the efflux of cholesterol induced by APLs altered cholesterol levels in HepG2 cells and, if so, whether the effects were reversible by cholesterol replenishment. As stated under *Results* and in accordance with reports by other authors using different cells lines (Li et al., 2006; Park et al., 2009), the exposure of HepG2 cells to 5 mM MβCD significantly depletes their cholesterol content and at the same time causes them to become round in shape. These changes are reversed by cholesterol replenishment. It is noteworthy that, although APLs also stimulate a depletion of cellular cholesterol and a similar morphological change, these effects are not fully reversed by the addition of exogenous cholesterol.

Several lines of study have demonstrated that depletion of cholesterol from the plasma membrane causes the disruption of lipid rafts and the consequent release of some of their constituents into non–raft-membrane domains, which renders them nonfunctional. Among the protein components whose activities have been proven to be modulated by asso-

ciation/dissociation to rafts, we were most interested in Akt. Other authors have reported alterations in the activity of this protein after treatment with perifosine and other APLs (Engel et al., 2008), although the mechanism involved in this effect is not yet fully understood. Because cholesterol is an essential lipid component of the rafts involved in Akt activation and, as we have demonstrated here, cholesterol is depleted by exposure to APLs, we looked to see whether changes in plasma-membrane cholesterol levels altered the activation state of Akt. We used MβCD as a positive control and found that incubation with MβCD decreased the phosphorylation of Akt and that, after replenishment with cholesterol, the levels of phosphorylated Akt returned to control values, all of which are in accordance with data reported in other cell lines after treatment with cyclodextrins (Park et al., 2009). Likewise, the depletion of cholesterol produced after exposure to APLs led to a reduction of Akt phosphorylation in HepG2 cells, suggesting that APLs may act on Akt via the depletion of plasma-membrane cholesterol. Moreover, in this case, the addition of cholesterol did not fully replenish cholesterol levels and did not restore the phosphorylation of Akt to control values. Thus, we propose that the depletion in plasma-membrane cholesterol caused by the efflux of cholesterol to the APL acceptor may be one of the underlying mechanisms for Akt inactivation reported previously by other authors.

Although further studies are needed, a common final mechanism for the APLs seems to underlie all of our observations; namely that the aqueous aggregates of APLs lead to a displacement of cholesterol from the plasma membrane, which can destabilize membrane microdomains and disturb their function in tumor cells. Thus, although APLs may act as an acceptor, as does MβCD, which binds cholesterol with high specificity, some amphiphilic compounds could insert themselves into the membrane, leading a displacement of cholesterol (Lange et al., 2009). Thus, we cannot rule out the possibility that amphipathic APLs might also act via a similar mechanism or even that both mechanisms might act cooperatively in the plasma-membrane cholesterol efflux. Thus, here we describe a new property of antitumoral APLs, those acting as efficient agents to deplete membrane cholesterol. Because these APLs are widely used as chemotherapeutic and antimicrobial drugs, the effect described in this article should be taken into account in experimental situations or clinical administration.

Acknowledgments

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Authorship Contributions

Participated in research design: Marco, Segovia, and Carrasco.

Conducted experiments: Ríos-Marco, Marco, Jiménez-López, and Carrasco.

Performed data analysis: Ríos-Marco and Carrasco.

Wrote or contributed to the writing of the manuscript: Ríos-Marco, Jiménez-López, Marco, Segovia, and Carrasco.

Other: Carrasco acquired funding for the research.

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4.6. Lipid efflux mediated by alkylphospholipids in HepG2 cells

In this work cholesterol efflux caused by APLs is analyzed more deeply. Firstly, we worked in serum-containing media in order to check if in this context APLs are able to promote cholesterol efflux. It is remarkable that when the cells are incubated with APLs in presence of serum the capacity to stimulate the efflux of cholesterol is suppressed, being the sorting rate the same in presence or absence of APLs.

On the other hand, there were neither significant differences between inhibition of cholesterol transport from plasma membrane to ER caused by APLs in either presence or absence of serum. This data, suggest that APLs are able to impair cholesterol arrival to ER, independently of their ability to promote cholesterol sorting. All these results reinforce the idea that the interference of APLs with the intracellular traffic of cholesterol is not directly related to cholesterol efflux.

On the other hand, we performed different experiments in presence of extracellular mixtures of APL:cholesterol and exposed HepG2 cells to them. Here we show that when the APL:cholesterol ratio is high, the system acts as cholesterol acceptor, whereas in cholesterol enriched vesicles, these complexes become donors. Thus, molar ratios from 1:40 (APL:cholesterol) are able to incorporate cholesterol into cells, instead of depleting them.

By using differential exposure-time radiolabeling, we marked cholesterol of non raft domains or the whole plasma membrane and measure its release to HePC. We found out that, while HePC impairs cholesterol transport to ER coming from both raft and non raft domains, the cholesterol efflux promoted by this agent seems to belong mainly to non raft regions.

As cholesterol metabolism is well co-ordinated with the homeostasis of choline-bearing phospholipids we also studied the efflux of these lipids. In addition to the efflux of cholesterol, APLs also stimulate the release of radiolabeled phosphatidylcholine and sphingomyelin from HepG2 cells to the medium. Interestingly, this release seems to follow that of the cholesterol.

In summary, it is important to note that when working in free serum solutions APLs may not just accept cholesterol from cell membrane but may also remove other membrane components, such as phospholipids. This could result in marked alterations to the permeability and structure of the cell membrane which may contribute to the antitumoral effects reported of APLs. In addition, APLs must be considered not only as cholesterol releasing agents but also as molecules that can act as efficient cholesterol donors.

Lipid Efflux Mediated by Alkylphospholipids in HepG2 Cells

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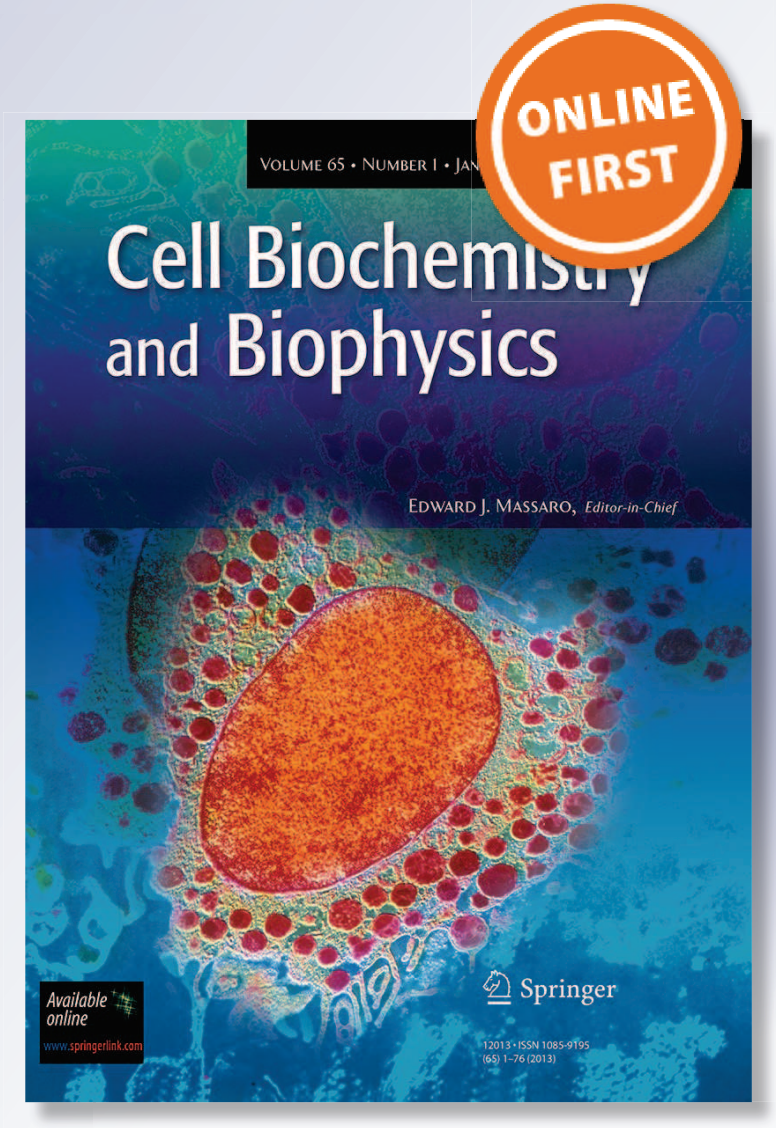

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ORIGINAL PAPER

Lipid Efflux Mediated by Alkylphospholipids in HepG2 Cells

Pablo Ríos-Marco · Josefa L. Segovia · José M. Jiménez-López · Carmen Marco · María P. Carrasco

Abstract Antitumoural alkylphospholipid (APL) analogues alter cholesterol homoeostasis in HepG2 cells by interfering with cholesterol transport from the plasma membrane to the endoplasmic reticulum (ER) and at the same time stimulating the release of considerable quantities of membrane cholesterol. The capacity of APLs to stimulate cholesterol efflux is suppressed when cells are incubated simultaneously with APLs and serum whilst the inhibition of cholesterol transport to the ER (measured in terms of the synthesis of esterified cholesterol) persists, indicating that both effects are independent of each other. Interestingly, our results suggest that both raft and non-raft membrane domains contribute to the cholesterol released to APLs. In addition, a marked efflux of choline-bearing phospholipids (phosphatidylcholine (PC) and sphingomyelins (SM)) was found to be related to this release of cholesterol. Finally, we observed that APL micelles composed of cholesterol might act as donor/acceptor cholesterol systems. Thus, the findings of this study clearly demonstrate that antitumoural APLs act as extracellular acceptors, stimulating cholesterol and phospholipid efflux, although they may also play a role as cholesterol donors.

Keywords Alkylphospholipids · Lipids · Efflux · Cholesterol acceptor · Cholesterol donor · HepG2

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Abbreviations

APL	Alkylphospholipid
CDs	Cyclodextrins
ER	Endoplasmic reticulum
ErPC	Erucylphosphocholine
FBS	Foetal bovine serum
HePC	Hexadecylphosphocholine
PC	Phosphatidylcholine
SM	Sphingomyelins
SMase	Sphingomyelinase

Introduction

Alkylphospholipid (APL) analogues are a new class of antitumoural agents that do not target DNA but insert themselves into the plasma membrane and subsequently trigger a broad range of biological effects, which ultimately lead to cell death [1, 2]. One important characteristic of APLs is their amphiphilic properties, which enable them to interact with cell membranes and affect cell metabolism at different levels [3]. Within this context, studies carried out in our laboratory have demonstrated that hexadecylphosphocholine (HePC), also known as miltefosine, edelfosine, perifosine and erucylphosphocholine (ErPC), alters intracellular cholesterol trafficking and metabolism, all of which leads to a cholesterogenic response involving increased gene expression and an enhanced uptake, synthesis and accumulation of cholesterol in the cell [4–6]. We have recently described a new property of antitumoural APLs: that of efficiently depleting membrane cholesterol [7], thus proving for the first time that APLs may act as extracellular acceptors. Thus, their acting as extracellular

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acceptors may constitute an important factor in determining the early biological activity of APLs.

Mammalian cells produce their own cholesterol and also receive cholesterol by uptake from lipoproteins. At the same time they continuously release cholesterol into the blood stream. Cell cholesterol homoeostasis is stringently controlled, its levels being maintained by a balance between uptake, efflux and endogenous synthesis. Many aspects of its metabolism are well known, including its uptake, its synthesis in the ER and its regulation. Nevertheless, though widely investigated, some doubt remains as to how cholesterol molecules move from the plasma membrane to extracellular acceptors. The generally recognised mechanism is now thought to involve the transfer of cholesterol molecules from the cell membrane through the aqueous phase to the acceptor particle, that is to say, by an aqueous diffusion mechanism. Other mechanisms have also been proposed, however, including collision or receptor mediation [8]. A significant fraction of the cholesterol that leaves the cell uses the simplest of homoeostatic mechanisms: equilibration driven by the difference in concentration between the plasma membrane and circulating lipoproteins. This passive efflux relies upon both uncatalysed and facilitated pathways. An example of the latter is export mediated by the plasma membrane scavenger receptor, SR-BI [9, 10] whilst active movement is driven by several members of the large family of plasma membrane ABC proteins [11].

The mechanism of hepatic cholesterol efflux has received little attention in the past because it has commonly been accepted that efflux is a matter of extra-hepatic cells. Nevertheless, efflux from hepatic cells deserves to be studied in greater detail because evidence is emerging that a large part of the pool of cholesterol released from cells comes from the liver [reviewed in [12]]. Having in mind the previously demonstrated effectiveness of APLs in inducing efflux of cholesterol from plasma membrane and that the interactions of APLs on membrane lipids could be quite complex, we have extended our studies to analyse further the characteristics of cholesterol-induced efflux to clinically relevant APLs. Thence our aim is to deeply investigate, in the HepG2 human hepatoblastoma cell line, the actions by which the APLs promote cholesterol release from the cell and analyse this process from different membrane domains and varied extracellular cholesterol scenarios. We observed that APLs stimulated the efflux of cholesterol from the plasma membrane, with both raft and non-raft domains contributing to the process, but that this release of cholesterol was not related to the impairment of cholesterol traffic from the plasma membrane to the ER, which triggers the cholesterogenic response. Our results also demonstrate that not only cholesterol but also phospholipids were released from the cell in the presence of APLs, although with different kinetics, the efflux of phosphatidylcholine (PC) and sphingomyelins (SM) following the release of cholesterol. Finally, we found that APLs can act as both acceptor and donor cholesterol systems, depending upon the APL/exogenous cholesterol molar ratios.

Materials and Methods

Chemicals and Reagents

Foetal bovine serum (FBS) was from The Cell Culture Company (Pasching, Austria). Minimal essential medium (MEM), sphingomyelinase (SMase), cholesterol and TLC plates were from Sigma-Aldrich (Madrid, Spain). [1,2-^{3}H(N)]cholesterol and [methyl-^{14}C]choline were from Perkin Elmer (Massachusetts, USA). HePC was from Cayman Chemical (Ann Arbor, MI, USA), edelfosine from Calbiochem (Nottingham, UK), ErPC from Alexis Biochemicals (Exeter, UK) and perifosine from Selleck Chemicals (London, ON, Canada).

Cell Culture

The HepG2 human hepatoblastoma cell line was from The European Collection of Animal Cell Cultures (Salisbury, UK). Cells were cultured in MEM containing 10 % heat-inactivated FBS supplemented with 2 mM L-glutamine, 1 % non-essential amino acids, 100 U/mL penicillin and 100 μg/mL streptomycin. Cells were grown in a humid atmosphere with 5 % CO_2 at 37 °C and subcultured at a ratio of 1:10 once a week. They were plated on 12-well plates (Nunc^{TM}) at a density of 5×10^4 cells/cm^2 and kept in culture medium before use in experimental assays at approximately 70 % confluence.

Cholesterol Efflux Assays

HepG2 cells were cultured in MEM/10 % FBS. The medium was removed and the cells were washed twice with PBS. To label the plasma membrane, radioactive [1,2-^{3}H(N)]cholesterol (2 μCi/ml) was added to the cells in a serum-free medium for periods of 15 or 60 min. To remove any unincorporated radioactivity the cells were washed three times with PBS containing 0.5 % BSA, and twice with PBS (both pre-warmed to 37 °C). APLs were added to the cells in a serum-free medium and aliquots were collected at various intervals from 0–90 min. Radiometric measurements of these aliquots were made by liquid scintillation using a Beckman 6000-TA counter (Madrid, Spain) and the percentage of efflux was calculated by the quantity of labelled cholesterol recovered in the

medium divided by the total radioactivity in the medium plus cells. Background (control) values were subtracted from treatment values. [13].

Phospholipid Efflux Assays

Radioactive [methyl-^{14}C]choline (1 μCi/ml) was added to a MEM/10 % FBS culture for a period of 36 h to label cells to equilibrium. To remove any unincorporated radioactivity the cells were then washed three times with PBS. APLs were added to the cells in a serum-free medium and aliquots were collected at various intervals from 0 to 60 min. Radiometric measurements of the aliquots were made by liquid scintillation using a Beckman 6000-TA counter (Madrid, Spain). Efflux of total phospholipids was calculated by the quantity of labelled phospholipids recovered from the medium divided by the total radioactivity in the medium plus cells and expressed as nmol/mg protein.

To examine the efflux of PC and SM, after 60 min treatment the medium was recovered and lipids from the cells and medium were extracted following the procedure of Bligh and Dyer [14]. PC and SM were separated by thin layer chromatography using a chloroform:methanol:acetic acid:H2O (60:30:8:5 v/v) mixture as solvent. Radiometric measurements of scraped lipid spots, rendered visible by exposure to iodine vapour, were made by liquid scintillation, and the percentage of efflux was calculated by the quantity of labelled PC or SM recovered from the medium divided by the total radioactivity of each phospholipid in the medium plus cells.

Trafficking of Cholesterol from the Plasma Membrane to the Endoplasmic Reticulum

One convenient way of measuring cholesterol transport from the plasma membrane to the ER is to determine the degree of esterification of radiolabelled cholesterol incorporated into the plasma membrane [6, 15]. To this end HepG2 cells were incubated with 2 μCi/ml of [1,2-^{3}H(N)]cholesterol for 15 and 60 min at 37 °C. To remove any unincorporated radioactivity the cells were washed three times with PBS containing 0.5 % BSA, and twice with PBS (both pre-warmed to 37 °C). The cells were then incubated for 1 h at 37 °C in MEM with or without 10 % FBS in the absence or presence of HePC, edelfosine, ErPC or perifosine, as described by Marco et al. [6]. After 60 min exposure the medium was removed, the cells washed with PBS and the lipids extracted following the procedure of Bligh and Dyer [14]. Cholesterol and cholesteryl esters were separated by TLC using a mixture of hexane:diethyl ether:acetic acid (70:30:2) as solvent. Radiometric measurements of scraped lipid spots, rendered visible by exposure to iodine vapour, were made by liquid scintillation. The fraction of esterified plasma membrane cholesterol is expressed in terms of the percentage of esterification of the total labelled cholesterol.

Influence of Alkylphospholipids in the Uptake of Exogenous Cholesterol Mediated in HepG2 Cells

APL/cholesterol mixtures were prepared using the following protocol: a volume of 30 μl of cholesterol from a stock solution of [1,2-^{3}H(N)]cholesterol (18.4 μM; 54.2 Ci/mmol) in ethanol was put into a glass tube. 20 μl of the concentration of aqueous APL required to give an APL/cholesterol molar ratio in the range of 1/2–1/80) was added and the tube was shaken thoroughly. 10 ml of MEM was then added and the prepared medium was used for the experiments. The final ethanol concentration in the medium never exceeded 50 mM, a quantity that has no effect on HepG2 cells, as we have previously reported [16].

When the cells were approximately 50 % confluent the medium was removed and the monolayers were washed three times with PBS containing 0.5 % BSA, and twice with PBS (both pre-warmed to 37 °C), after which the APL/cholesterol solutions in MEM were added. After 120 min incubation the medium was removed and the cell monolayer washed three times with cold PBS. Lipids were extracted from the cells as described above and the quantity of radiolabelled cholesterol incorporated into the cells was determined by liquid scintillation counting.

Statistics

Results are expressed as mean ± SEM. A one-way ANOVA with post hoc comparisons by Scheffe's test was carried out (SPSS 13.0). P values < 0.05 were considered to be statistically significant.

Other Analyses

Cell-protein content was determined in the cell homogenates by Bradford's method [17] using BSA as standard.

Results

Cholesterol Efflux to Extracellular Acceptors

Recent studies in our laboratory have demonstrated the capacity of several APLs to promote cholesterol efflux from the plasma membrane to the medium by acting as extracellular cholesterol acceptors, in an order of efficiency of edelfosine > HePC > perifosine > ErPC [7]. In this work we have extended our previous investigations to analyse more thoroughly the characteristics of cholesterol-induced efflux. We first compared the efflux of cholesterol

to complete serum, the most complex and yet the most physiological cholesterol acceptor, with the efflux of cholesterol to HePC (as a representative of the APLs). The difference in the kinetics of cholesterol efflux to both extracellular acceptors can be seen in Fig. 1. Incubation of HepG2 cells with 50 μM HePC in a serum-free medium resulted in the release of 25 % of the plasma membrane cholesterol during the first 10 min′ incubation, whilst in cells incubated with 10 % FBS the cholesterol was released at a roughly constant rate throughout the incubation, with 20 % having been released after 90 min. (All data of HePC show significant differences regarding to FBS samples, $P < 0.001$). It turned out that in the presence of 10 % FBS none of the APLs tested exerted any significant effect on the release of cholesterol from the plasma membrane, as compared with cholesterol output in the presence of 10 % FBS alone, represented in Fig. 2 as a dashed horizontal line.

Cholesterol Traffic from the Plasma Membrane to the Endoplasmic Reticulum

We also studied the effects of APLs on the traffic of cholesterol from the plasma membrane to the ER under conditions of enhanced cholesterol efflux, i.e. after exposure to APLs alone and after exposure to an APL in a medium containing whole serum, in which the APL does not stimulate cholesterol output. In the belief that the best experimental procedure to study any interference in the traffic of cholesterol from the plasma membrane to the ER is to analyse the rate of cholesterol ester synthesis from plasma membrane cholesterol [6, 15] we labelled plasma membrane with radioactive cholesterol for 60 min. Labelled cells were exposed to the different APLs, both in the presence and absence of 10 % FBS, and the radioactivity appearing in the esterified cholesterol was determined. As reported in a previous publication [18], all the APLs assayed impeded the arrival of plasma membrane cholesterol to the ER and thus its subsequent esterification, but this interference was also observed in the presence of serum, where we found no stimulation of cholesterol efflux by APLs (Fig. 3, where $^{*}P < 0.05$; $^{**}P < 0.005$ compared to the corresponding control).

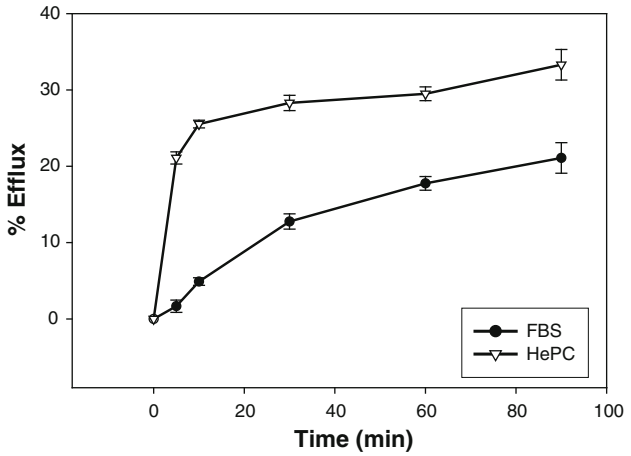

Fig. 1 Cholesterol efflux to the extracellular serum and to hexadecylphosphocholine. Log-phase HepG2 cells were grown for 24 h in MEM containing 10 % FBS. Cells were washed with PBS and then incubated for 60 min in 2 μCi/ml of [1,2-^{3}H(N)]cholesterol in serum-free MEM. They were then washed with PBS containing 0.5 % albumin and PBS at 37 °C and subsequently treated for 90 min in MEM without any additions (control) or with 50 μM HePC or 10 % FBS. Aliquots of medium were collected after 5, 10, 30, 60 and 90 min. Radioactive cholesterol was measured as described in Materials and Methods. Cholesterol efflux is expressed in terms of percentage as radioactive cholesterol in medium/total radioactive cholesterol and represents the mean ± SEM of three independent experiments conducted in triplicate. All data of HePC show significant differences regarding to FBS samples, $P < 0.001$

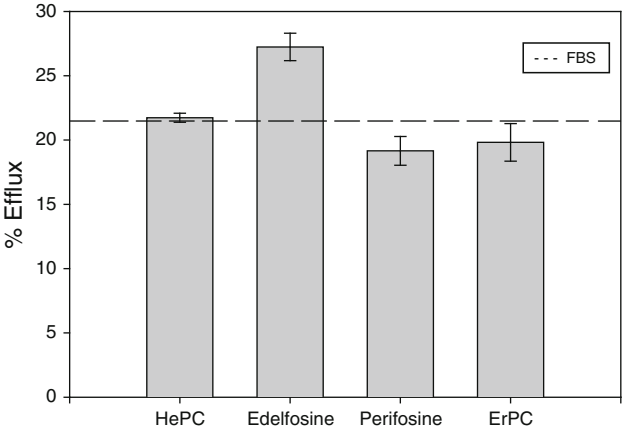

Fig. 2 Cholesterol efflux after the simultaneous addition of alkylphospholipids and serum. Log-phase HepG2 cells were grown for 24 h in MEM containing 10 % FBS. Cells were washed with PBS and then incubated for 60 min in 2 μCi/ml of [1,2-^{3}H(N)]cholesterol in serum-free MEM. They were then washed with PBS containing 0.5 % albumin and PBS at 37 °C and subsequently treated for 90 min in MEM without any additions (control) or with 50 μM HePC, edelfosine, perifosine or ErPC in the presence of 10 % FBS. Aliquots of medium were collected at the end of the assay. Radioactive cholesterol was measured as described in Materials and Methods. Cholesterol efflux is expressed in terms of percentage as radioactive cholesterol in medium/total radioactive cholesterol and represents the mean ± SEM of three independent experiments conducted in triplicate

Effects of Hexadecylphosphocholine and Sphingomyelinase on the Efflux and Traffic of Cholesterol from the Plasma Membrane to the Endoplasmic Reticulum

In further experiments we decided to label plasma membrane cholesterol for both 15 and 60 min and to determine the effects of HePC and SMase on both the efflux and esterification of plasma membrane cholesterol. As far as

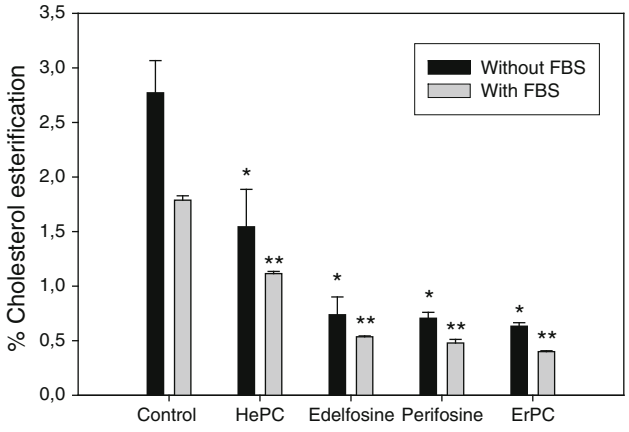

Fig. 3 Cholesterol traffic from the plasma membrane to the endoplasmic reticulum after the simultaneous addition of alkylphospholipids and serum. Log-phase HepG2 cells were grown for 24 h in MEM containing 10 % FBS. Cells were washed with PBS and then incubated for 60 min in 2 μCi/ml of [1,2-^{3}H(N)]cholesterol in serum-free MEM. They were washed with PBS containing 0.5 % albumin and PBS at 37 °C and then treated for 60 min with 50 μM HePC, edelfosine, perifosine or ErPC; or else 50 μM of each alkylphospholipid plus 10 % FBS. Radioactive cholesterol was measured as described in Materials and Methods. The esterified fraction of plasma membrane cholesterol is expressed in terms of the percentage of esterification of the total labelled cholesterol and represents the mean ± SEM of three independent experiments conducted in triplicate ($^{*}P < 0.05$; $^{**}P < 0.005$ compared to the corresponding control)

cholesterol traffic is concerned, exposure to HePC of the cells labelled with [1,2-^{3}H(N)]cholesterol for 60 min decreased the esterification of cholesterol by up to 67 % of the levels of control cells (Table 1). As expected, in cells incubated with SMase we observed a significant increase in the esterification of plasma membrane cholesterol due to its release from membrane domains rich in SM/cholesterol (rafts). It is noteworthy that when cells were exposed to HePC plus SMase we found a very substantial reduction in the esterification of cholesterol coming from the plasma membrane. When cells were labelled with [1,2-^{3}H(N)]cholesterol for only 15 min, their exposure to HePC resulted in a significant decrease of up to 64 % in cholesterol esterification, whilst surprisingly, in cells incubated with SMase the synthesis of cholesterol esters remained unaltered. According to these effects in cells incubated in the presence of HePC plus SMase, the formation of cholesterol ester is considerably inhibited when plasma membrane cholesterol is labelled for 15 min.

We then undertook an analysis of the consequences that SM hydrolysis has on cholesterol efflux induced by HePC. In both the 15- and 60-min labelling protocols HePC stimulated a substantial release of cholesterol into the extracellular medium (Fig. 4). It can also be seen that when cells were incubated in the presence of both HePC and SMase the same percentage of cholesterol was present in the extracellular medium, and thus a similar cholesterol efflux occurred as that found after incubation with HePC alone.

Kinetics of the Efflux of Choline-Containing Phospholipids

To determine whether cholesterol efflux induced by HePC was accompanied by the release of PC and SM, the main phospholipid constituents of rafts, HepG2 cells were labelled to equilibrium with [methyl-^{14}C]choline, and the time course of the release of radioactivity into the medium was determined throughout a 60-min incubation period in the presence of HePC. The release of choline-bearing phospholipids followed a clearly different kinetic from that of cholesterol (Fig. 5a). Thus, whilst the kinetic of cholesterol efflux was bi-exponential and a high fraction of cholesterol was released in the first minutes of incubation, the quantity of PC plus SM released into the medium increased gradually and in fact the efflux of both phospholipids had still not reached a plateau after the full incubation period. These results suggest that cholesterol is rapidly released to the APL acceptor and that the efflux of PC and SM follows the release of cholesterol. With regard to the individual phospholipids, we also determined the level of radioactive PC and SM released into the medium at the end of incubation. Exposure of the HepG2 cells to the APL resulted in a significant release of PC since approximately 9 % of the total PC was recovered in the medium (Fig. 5b). In addition, SM was also detected in the extracellular medium, although to a considerably lesser extent, since only 2 % of this phospholipid was released from the cell by HePC activity. In any case the release of PC and SM was significantly increased with HePC ($^{*}P < 0.001$).

Alkylphospholipids as Cholesterol Donor/Acceptor System

It has been reported that several molecules such as CDs can act as either cholesterol acceptor or donor systems. Although APLs are structurally very different from CDs we went on to find out whether APLs might, in combination with cholesterol, also act as a cholesterol donor system. To this end we exposed HepG2 cells to different quantities of HePC (as a representative of APLs in general) and the same quantity of [1,2-^{3}H(N)]cholesterol to give APL/cholesterol molar ratios ranging from 1/2 to 1/80. As can be seen in Fig. 6a, in the presence of very low levels of HePC (1 μM) with an APL/cholesterol molar ratio of 1/80, the HepG2 cells incorporated substantially more extracellular cholesterol than that observed in cells exposed to the same quantity of cholesterol in the absence of the APL (horizontal dashed line), demonstrating that this APL can indeed act as cholesterol donor. Interestingly, when we incubated

Table 1 Effects of hexadecylphosphocholine and sphingomyelinase upon the trafficking of cholesterol from the plasma membrane to the endoplasmic reticulum

	15 min	60 min
Control	0.50 ± 0.10	2.73 ± 0.19
HePC	0.18 ± 0.02*	0.89 ± 0.05*
SMase	0.42 ± 0.04	3.67 ± 0.09*
HePC + SMase	0.10 ± 0.02*	0.81 ± 0.04*

Log-phase HepG2 cells were grown for 24 h in MEM containing 10 % FBS. Cells were washed with PBS and then incubated for 15 or 60 min in 2 μCi/ml of [1,2-^{3}H(N)]cholesterol in serum-free MEM. They were then washed with PBS containing 0.5 % albumin and PBS at 37 °C and subsequently treated for 60 min without any additions (control) or with 50 μM HePC or 100 mU/ml SMase. The esterified fraction of plasma membrane cholesterol is expressed in terms of the percentage of esterification of the total labelled cholesterol and represents the mean ± SEM of three independent experiments conducted in triplicate

* $P < 0.04$ compared to control values

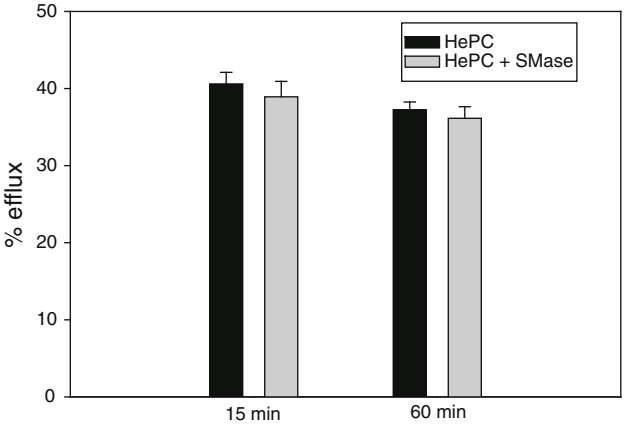

Fig. 4 Comparative effect of hexadecylphosphocholine and sphingomyelinase on cholesterol efflux. Log-phase HepG2 cells were grown for 24 h in MEM containing 10 % FBS. Cells were washed with PBS and then incubated in 2 μCi/ml of [1,2-^{3}H(N)]cholesterol in serum-free MEM for 15 or 60 min. They were then washed with PBS containing 0.5 % albumin and PBS at 37 °C and subsequently treated for 60 min without any additions (control) or with 50 μM HePC or 50 μM HePC and 100 mU/ml SMase. Aliquots of medium were collected at the end of each assay. Radioactive cholesterol was measured as described in Materials and Methods. Cholesterol efflux is expressed in terms of percentage as radioactive cholesterol in medium/total radioactive cholesterol and represents the mean ± SEM of three independent experiments conducted in triplicate. Background (control) values were subtracted from treatment values

the cells with the same quantity of cholesterol but increasing quantities of HePC, the incorporation of exogenous cholesterol into the cell decreased gradually, and in fact in the presence of molar ratios lower than 1/10 the cells incorporated considerably lower quantities of cholesterol than cells that had not been exposed to this APL. Bearing in mind the potential importance of this result, we decided to check that this effect was not specific to HePC by repeating the experiment with edelfosine. The results shown in Fig. 6b confirm that the profile of cholesterol incorporation into the cell strongly reproduces that observed with the HePC/cholesterol mixtures:edelfosine/cholesterol molar ratios ranging from 1/20 to 1/40 were able to stimulate the incorporation of cholesterol into HepG2 cells, the effect being more marked the lower the APL/cholesterol molar ratio.

Discussion

In previous studies we have demonstrated that antitumoural APLs alter cholesterol homoeostasis by inhibiting cholesterol transport from the plasma membrane to the ER in HepG2 cells [3, 18]. We also described for the first time that APLs may act as extracellular acceptors of cholesterol molecules with a kinetic which mimics that observed with other well known exogenous cholesterol acceptors such as cyclodextrins (CDs) [7], oligosaccharides that have a higher affinity for sterols than other lipids, which makes them quite effective in modifying cholesterol metabolism in vivo [19].

The purpose of this present investigation was to study the role of different APLs such as HePC, edelfosine, perifosine and ErPC in mediating cell cholesterol efflux to gain further understanding of the characteristics of APLs in releasing cholesterol from the cells. Thus we first compared the capacity of APLs to stimulate cholesterol efflux compared to whole serum as extracellular cholesterol acceptor. The choice of whole serum as acceptor is important to establish the lipid, lipoprotein and serum proteins that most govern cholesterol efflux under physiological conditions. When we compared cholesterol efflux mediated by APLs and by whole serum our results demonstrated that 50 μM HePC stimulated the release of higher quantities of cholesterol than did 10 % whole serum. We also observed that APLs produced the release of most of the cholesterol within the first 10 min whilst the cholesterol efflux kinetic is different in cells incubated with serum, which results in a relatively constant rate of cholesterol release throughout the entire 90 min incubation. Similar results have been obtained by other authors for the kinetics of cholesterol output to CDs and HDL, respectively [20, 21]. It is noteworthy that when cells are incubated with APLs plus serum the capacity of the APL to stimulate the efflux of cholesterol is suppressed. These results can be explained in terms of a specific interaction between HePC and the cholesterol from serum lipoproteins, which would decrease the effective concentration of the APL [22].

In any case, although APLs do not stimulate any cholesterol efflux from cells when incubated in the presence of serum, all the APLs assayed produced a marked inhibition of cholesterol esterification from the plasma membrane as a consequence of the inhibition of sterol transport from the

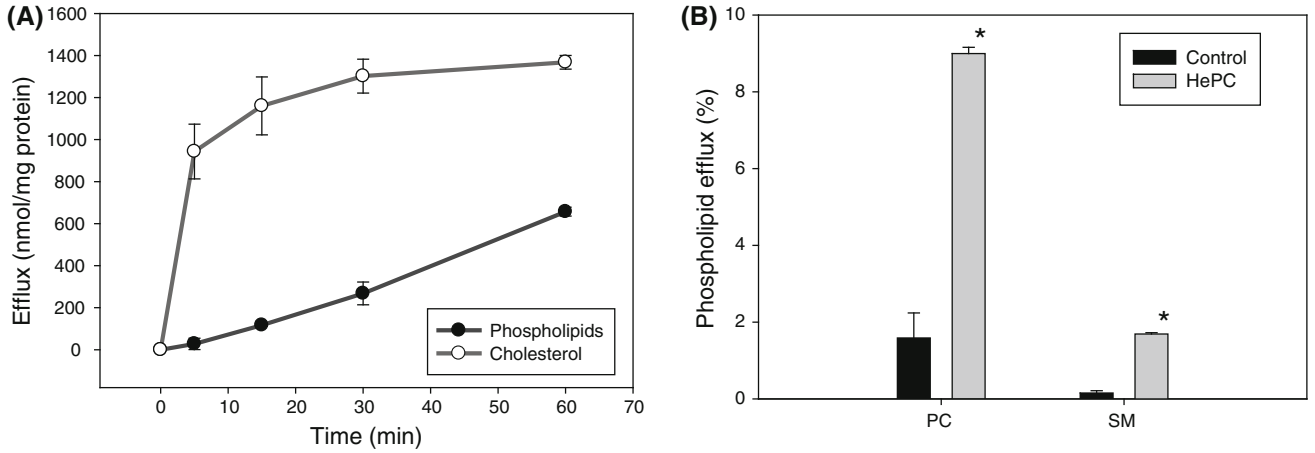

Fig. 5 Effect of hexadecylphosphocholine on the efflux of choline-bearing phospholipids. Log-phase HepG2 cells were grown for 24 h in MEM containing 10 % FBS. Cells were washed with PBS and then incubated for 36 h in 1 μCi/ml [methyl-^{14}C]choline in MEM/10 % FBS to label them to equilibrium. They were then washed with PBS containing 0.5 % albumin and PBS at 37 °C and subsequently treated for 60 min in MEM without any additions (control) or with 50 μM HePC. **a** Aliquots of the cell medium were collected after 5, 15, 30 and 60 min. Radioactive choline-bearing phospholipids were measured as described in Materials and Methods. Choline-bearing phospholipid efflux is expressed as nmol of radioactive choline-bearing phospholipids/mg protein and represents the mean ± SEM of three independent experiments conducted in triplicate. The time course of [1,2-^{3}H(N)]cholesterol efflux is also represented in the same figure for comparison. **b** Aliquots of the media from control or cells treated with 50 μM HePC were collected after 60 min and PC and SM were separated as described in Materials and Methods. Choline-bearing phospholipid efflux is expressed in terms of percentage of labelled PC or SM recovered from the medium divided by the total radioactivity in the medium plus cells of each phospholipid and represents the mean ± SEM of three independent experiments conducted in triplicate ($^{*}P < 0.001$ compared to the corresponding control)

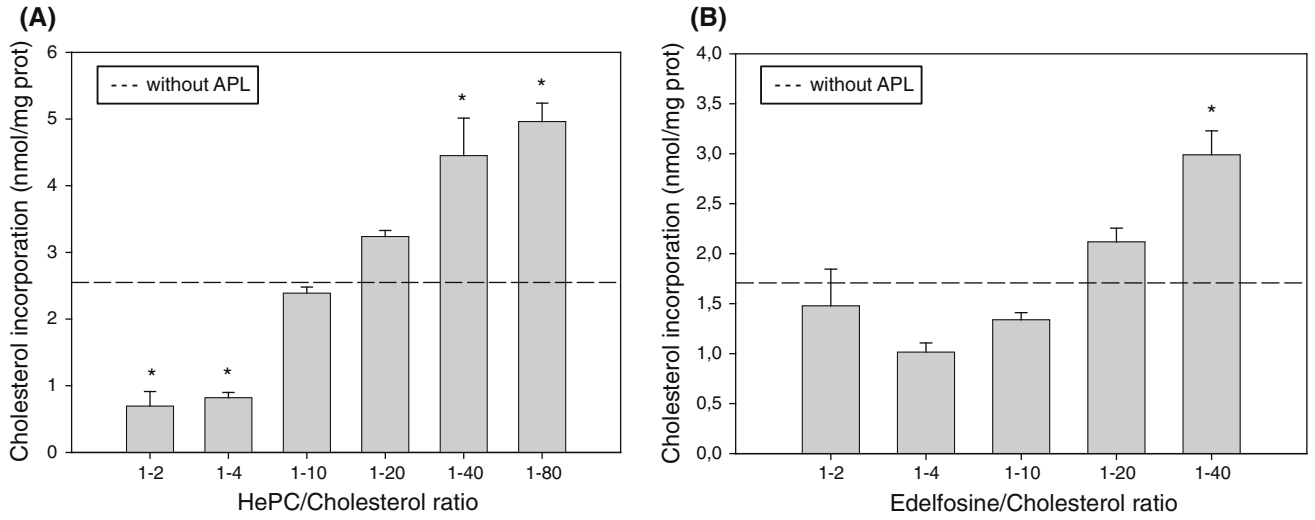

Fig. 6 Alkylphospholipids as cholesterol donor/acceptor systems. Log-phase HepG2 cells were grown for 24 h in MEM containing 10 % FBS. Cells were washed with PBS and then exposed to a mixture composed of 30 μl of cholesterol from a stock solution of [1,2-^{3}H(N)]cholesterol (18.4 μM; 54.2 Ci/mmol) plus different quantities of HePC **a** or edelfosine **b** to a final volume of 10 ml of MEM to give APL/cholesterol molar ratios ranging from 1/2 to 1/80. After 120 min incubation the medium was removed and the cell monolayer washed with PBS containing 0.5 % albumin and PBS at 37 °C. Lipids were extracted from the cells as described in Materials and Methods and the quantity of radiolabelled cholesterol was determined by liquid scintillation counting and expressed as nmol of radioactive cholesterol/mg protein. The results represent the mean ± SEM of three independent experiments conducted in triplicate ($^{*}P < 0.05$ compared to cholesterol-free APL assays)

plasma membrane to the ER. All these results reinforce the idea that the interference of APLs with the intracellular traffic of cholesterol is not related to cholesterol efflux because in the presence of 10 % FBS the APLs did not stimulate cholesterol efflux but esterification was significantly impaired.

It has been reported that HePC interacts strongly with cholesterol and other sterols, as do other APLs [7, 22, 23]

and thus in the past we have suggested two possible mechanisms to explain the activity of APL on plasma membrane cholesterol at the molecular level [7, 18]: (1) The aqueous aggregates of APLs could act as a system acceptor of cholesterol, which may lead to the displacement of cholesterol from the plasma membrane observed in the absence of serum; (2) As do other amphiphilic compounds, APLs could insert themselves into the membranes, thus retaining cholesterol and reducing membrane sterol reactivity, which would lead to a lower influx of sterol to the ER [24–26]. On the basis of our recent results we now hypothesize that both mechanisms could act simultaneously at the level of the plasma membrane. Thus, we propose that APLs interfere with cholesterol transport from the plasma membrane to the ER and in addition act as efficient acceptors of cholesterol when there are no other physiological acceptors such as serum.

There is evidence to suggest that within the plasma membrane cholesterol associates preferentially with choline-bearing phospholipids to form condensed, liquid-ordered (Lo) phases, these complexes being immiscible in the sterol-poor or liquid-disordered (Ld) phases. A preliminary proposal is that plasma membrane cholesterol exists in low- and high-activity states, i.e. low or high escape tendency, or fugacity [27, 28]. It has been proposed that cholesterol in Ld domains (non-raft) has a higher tendency to escape and must be more easily extracted in the presence of exogenous acceptors than that in Lo domains (raft). We reasoned that if this hypothesis was correct the labelling of plasma membrane cholesterol for a short time would mainly label the Ld membrane domains, whilst extending the period of cholesterol exposure would also label the Lo domains, and thus we would be able to determine from which membrane domain the cholesterol was released. Another fact we took into account is that the addition of neutral SMase is generally believed to lead to the hydrolysis of plasma membrane SM in rafts, thus disrupting the raft structure and causing the translocation of free cholesterol to ER, where it is esterified. Support for this comes from research that has demonstrated that treatment of living cells with SMase degrades the major raft sphingolipid in the plasma membrane and leads to a rapid increase in ER cholesterol, as evidenced by cholesterol ester production [29, 30].

Bearing all this in mind, we further investigated the source of cholesterol released by APLs. To do this we labelled plasma membrane with radioactive cholesterol for 15 and 60 min (see Materials and Methods) and determined the cholesterol esterification and efflux induced by APLs both in the presence and absence of SMase. With regard to cholesterol esterification, as might be expected, when the cells are labelled with cholesterol for 60 min and are treated with SMase, an increase in cholesterol esterification can be seen, whilst if plasma membrane is labelled with cholesterol for only 15 min, SM hydrolysis is not accompanied by any increase in cholesterol esterification. This unexpected result may be due to the fact that during this short period of time no cholesterol in any membrane pool accessible to SMase was labelled. Thus our data clearly point to the fact that the incorporation of radioactive cholesterol for 15 min only labels non-raft domains, whilst after 60 min both raft and non-raft fractions are labelled. To our knowledge, this is the first experimental evidence for such differential labelling of specific plasma membrane domains.

Our results also show that after both 15 and 60 min labelling exposure to HePC leads to a significant decrease in the esterification of cholesterol. These results accord with the capacity of APLs to interfere with the traffic of cholesterol from the plasma membrane to the ER, as we have reported in previous publications [7, 18]. Noticeably, the esterification of plasma membrane cholesterol was inhibited by HePC both in the presence and absence of SMase, demonstrating that the enhanced mobilization of cholesterol produced by SMase treatment did not reach the ER to be esterified due to its transport being impeded by HePC.

As far as cholesterol efflux is concerned, we found that after labelling for both 15 and 60 min, HePC stimulated a similar release of cholesterol (40 %) into the extracellular medium. That the fraction of cholesterol released was almost identical after both periods of labelling clearly points to the fact that both raft and non-raft fractions contribute to the cholesterol released to HePC. Interestingly the efflux of cholesterol to APLs was not affected after SM hydrolysis produced by SMase, that is to say, SM degradation did not lead to a cholesterol in the plasma membrane more susceptible to extraction by HePC. These results clearly contrast with those obtained by other authors who have reported that modifications in the SM content as a result of SMase treatment enhance cholesterol efflux to CDs [31, 32]. Our data indicate that APLs act in a different way from CD, which has been reported to release cholesterol from lipid rafts [33]. Nevertheless, it is evident that cholesterol is not confined to lipid rafts and it is even debatable whether CDs act exclusively in these lipid domains [34, 35]. On the contrary it seems likely to us and other authors [28, 35] that cholesterol is in fact similarly or even preferentially removed from non-raft domains of the plasma membrane where the lipids are less tightly packed.

Cholesterol homoeostasis is well co-ordinated with the homoeostasis of choline-bearing phospholipids. In addition to the efflux of cholesterol, APLs also stimulate the release of cell PC and SM into the medium. PC and SM present in the plasma membrane, mainly in the outer monolayer, are critical for maintaining the plasma membrane structure and function. A number of studies have reported only a minimal

release of phospholipids from cell membranes on exposure to CDs [20, 36]. Our results, on the other hand, demonstrate clearly the capacity of APLs to release plasma membrane cholesterol and also that there is a correlation between cholesterol and phospholipid efflux and that HePC stimulates a considerable efflux of both PC and SM. The main difference resides in that the kinetic of cholesterol efflux to HePC is bi-exponential, which agrees with the idea of two distinct plasma membrane cholesterol pools, whilst the kinetic of choline-bearing phospholipids is linear, suggesting the existence of a single kinetic pool for the efflux of these phospholipids. As remarked upon in the Results section, the fractional release of phospholipids is considerably less than that of cholesterol. The reason for this probably reflects the lower aqueous solubility of phospholipid compared to cholesterol, which would be consistent with an aqueous diffusion mechanism and the distribution of phospholipid in plasma and internal membranes, whereas cholesterol is enriched in the plasma membrane and thus more available. These results show for first time that APLs remove not only cholesterol but also phospholipids, although they preferentially release cholesterol.

A wide variety of extracellular molecules, such as CD, apolipoproteins, HDL and whole serum acceptors have been used in cholesterol flux studies. These acceptors differ in their structure, complexity and the extent to which they can act as donor and/or acceptor systems, thus contributing to either unidirectional or bidirectional flux [34, 37]. Other types of molecule that can act as cholesterol acceptors, recently described in our laboratory, are APLs, lipid analogues that have a high affinity for sterols. In the present study we have widened our previous investigations and used APL micelles, either cholesterol-free or prepared with cholesterol, to give different APL/cholesterol molar ratios.

We have shown that the exposure of HepG2 cells to pure APL micelles results essentially in the unidirectional movement of cholesterol from the plasma membrane to the extracellular lipid analogue, resulting in a reduction in the cholesterol mass [7] but it also means that cholesterol-bearing micelles are able to deliver molecular cholesterol to the cell, presumably to a single site, i.e. the plasma membrane. Our results suggest in fact that the ratio between the quantities of cholesterol and APL will determine whether it will act as cholesterol acceptor or donor. Our hypothesis is that the movement of cholesterol between the plasma membrane and the APL is bidirectional and depends upon the cholesterol concentration gradient, as it occurs with other molecules such as CD and phospholipid [37]. So, cholesterol-free APL micelles can accept molecules of this sterol desorbed from the plasma membrane. When APL micelles contain increasingly high cholesterol levels, however, the concentration gradient is reduced and thus the capacity to accept cholesterol from plasma membrane is reduced concomitantly. This is with all probability the reason for the previously described effect that the simultaneous addition of cholesterol with the APL significantly reduces its capacity to stimulate cholesterol efflux from plasma membrane [7]. When the APL/cholesterol molar ratio reaches a certain threshold value cholesterol is not released from the plasma membrane. At lower molar ratios, cholesterol molecules are desorbed from the surface of the micelles and thence are incorporated into the cell membrane. Whether this cholesterol movement is produced simply by aqueous diffusion and/or directed by some receptor, such as SRBI, which is involved in the bidirectional cholesterol movement, remains to be clarified in future studies.

In conclusion APLs have shown specific skills to extract cholesterol that has never been stated before. Cholesterol concentration has been shown to be determinant to promote either cholesterol efflux or donation at biological membranes. Differential cholesterol labelling in plasma membrane and posterior efflux by APLs has been described for first time too in this work. Thus, it is important to take into account that APLs may not just accept cholesterol from cell membrane but may also remove other membrane components, such as phospholipids, which could result in alterations to the permeability and structure of the cell membrane. The capacity of APLs to release cell cholesterol and phospholipids should be taken into account in experimental situations or clinical administration.

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4.7. Performing new tools for alkylphospholipids study: FRET microscopy and 3D cultures

This study corresponds with part of work that I carried out in 2011 during my research stay abroad in Bart´s Cancer Institute. This centre belongs to Queen Mary´s University of London, United Kingdom. This work was tutorized by Dr. Salma Taboubi, postdoctoral researcher in the Cell Signaling Department. This research group is one of the most internationally outstanding teams investigating the PI3K/AKT pathway. During four months of research, two new techniques were applied to study the influence of APLs on the PI3K/AKT pathway: Fluorescence Resonance Energy Transfer (FRET) and in vitro acini development through 3 dimensions cultures.

FRET was applied to study the levels of phosphoinositides inside cells. These levels related with PI3K activation status and can be essential for understanding of the AKT dependent cancer progression and its possible modulation by APLs.

On the other hand, artificial acini cultures raise the possibility of obtaining information, working in vitro, of physiological models that are similar to breast development. APLs have been shown some effectiveness against mammary cancer and inmunofluorescence of 3D systems provide one of the newest techniques in knowledge of cancer migration and cells polarization.

4.7.1 Analysis of phosphatidylinositol triphosphate production in perifosine-treated 4T1 cells

Introduction

Phosphoinositide 3-kinases (PI3Ks) are lipid kinases that phosphorylate the 3-hydroxyl on the inositol ring of phosphatidylinositol and its phosphorylated derivatives phosphatidylinositol 4,5-bisphosphate (PIP2) and phosphatidylinositol 4-monophosphate (PI4P). PI3Ks receive inputs from activated tyrosine kinase receptor and heterotrimeric guanine nucleotide–binding protein coupled receptors and produce lipid second messengers that affect cell proliferation, growth, metabolism, motility, and intracellular trafficking (Taboubi et al., 2010; Vadas et al., 2011). In terms of regulating cell division and tumorigenesis, the most important PI3K proteins are those that belong to class IA, with a catalytic subunit p110α and an associated regulatory subunit p85. In quiescent cells, the regulatory subunit p85 maintains the p110α catalytic subunit in a low-activity state (Hafsi et al., 2012). When PI3K is activated by receptor/ligand binding (e.g. Insulin like growth factor with its receptor), this kinase catalyze lipid PIP2 phosphorylation into phosphatidylinositol 3,4,5-triphosphate (PIP3) in plasma membrane. PIP3 production promotes AKT (PKB) transport to plasma plasma membrane, where is phosphorylated by PDK1 and mTOR for its activation (Kawauchi et al., 2009; Zhou and Huang, 2011). Phosphorylated isoform of PKB phosphorylates different substrates inducing cell survival (Zhou and Huang, 2011). Moreover PI3K/AKT pathway has been reported to be overexpressed in many cancer lines and for this reason is considered as an attractive target for cancer therapy (Wang et al., 2011; Xue et al., 2012).

However, PIP3 production by PI3K is a poorly known process. This is because of the lack of suitable methods to quantitatively analyze the spatial and temporal dynamics of PIP3 in living cells. Some methods as labeling with ^{32}P-ortophosphate have been tried. However, it has several limitations as millions of cells must be examined to obtain a valid signal. Fusion proteins of green fluorescent protein (GFP) and PIP3-binding domains have been tried to be used as indicators for PIP3 accumulation in the cellular membrane (Venkateswarlu et al., 1998; Varnai et al., 1999). However, several factors, such as membrane ruffles and shape alterations, affect the fluorescence intensity change in the region of interest causing misleading results.

To overcome these limitations, it has been developed fluorescent indicators for PIP3 based on FRET (Miyawaki and Tsien, 2000; Sato et al., 2002). In this work we study the effects that one of the APLs, the perifosine, exerts on PIP3 production by FRET microscopy.

Materials and Methods

In these assays a specific plasmid was used for measuring PIP3 levels by FRET microscopy. This plasmid encodes a protein with the following elements: 1) membrane localization sequence (MLS), 2) pleckstrin homology (PH) domain, 3) cyan fluorescent (CFP) variant protein, 4) yellow fluorescent (YFP) variant protein. PH domain joins specifically to PIP3 and is situated as a linker between CFP and YFP. When plasmid is expressed inside the cell, this protein goes to membrane and anchor to it, becoming then able to interact with PIP3 through of its PH domain. When this union occurs there is a "flip-flop-type" conformational change that allows nanometer proximity between CFP and YFP. CFP excitation at 440 nm produces energy emission at 480 nm that promotes both excitation and emission of fluorofore YFP, but only when these two elements are interacting. So, it is necessary PIP3 presence for YFP excitation at 440 nm, otherwise there is not contact nor activation of YFP (Fig. 1).

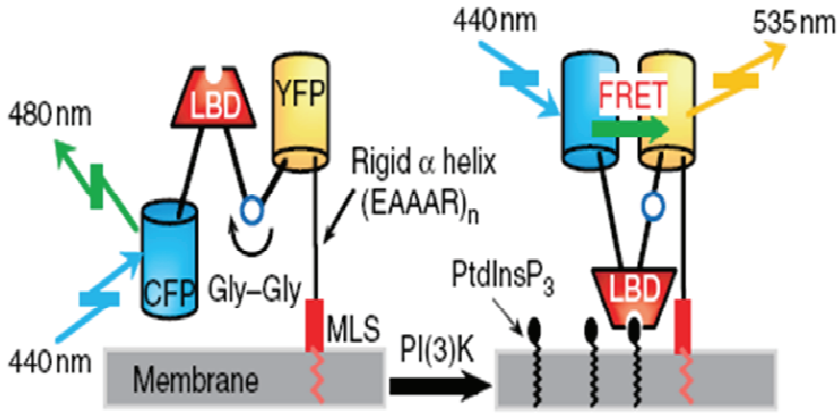

Figure 1. CFP and YFP interaction in FRET probes. PI3K phosphorylates PIP2 to PIP3 ($PtdInsP_3$). FRET poliprotein associates to PIP3 through the pleckstrin homology binding domain (PH)/(LBD) which allows conformational change that make CFP and YFP interact (right side). There is no possible FRET interaction without PIP3 (left side). From Sato et al., 2003.

We used two FRET probes with the only difference of the MLS region. These MLS are directed against plasma membrane (FRET-PM) or endomembranes (FRET-EM). The selected cell line for this study was the 4T1 breast cancer cell from mouse. Cells were seeded in 10 cm plates and allowed to grow for 24 h. 4T1 cells were transfected with FRET-PM or FRET-EM. 24 h later cells were reseeded over sterile coverslips in cell culture plates and allowed to adhere. They were treated with MEM containing 10 µM perifosine for 0, 30 or 90 min. After this time they were fixed with 3.7% formaldehyde for 15 min, rinsed 3 times with PBS and mounted over microscopy slides using Mowiol.

Sample analysis was done by confocal microscopy. Using the suitable software, cells were excited and CFP emission was measured. After this, YFP bleaching was done and CFP emission was checked again. Both values were used to calculate the FRET index (i.e fluorofores interaction) which is: FRET index = CFP emission after bleaching/ CFP emission before bleaching (Fig. 2).

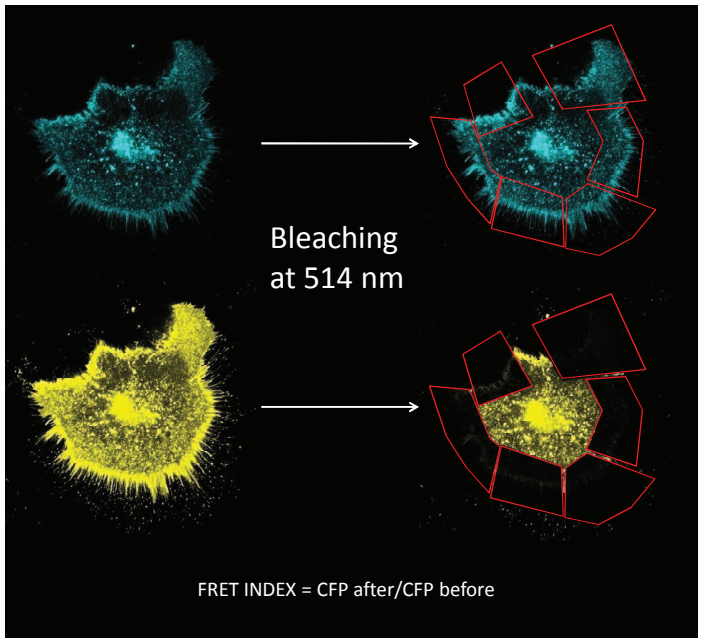

Figure 2. Bleaching of YFP and FRET index calculation. Emission of CFP and YFP was measured in 4T1 cells (left side). After this, bleaching at 514 nm was done in different cells regions (right side, red sections). Bleaching showed a visual decrease in YFP intensity but not in CFP. After bleaching, CFP emission was measured again for FRET index calculation.

Results

Using this technique we were able not only to quantify the PIP3 levels but its location (plasma membrane or endomembranes). The time-course obtained from 0 to 90 min of perifosine exposition allowed us analyze modification of PIP3 levels as a dynamic effect that relates different membranes. So, we observed that levels of PIP3 in plasma membrane remained unaltered after 30 min compared to the control (0 min exposure). However, after 90 min there was a decrease in these levels (Fig. 3A). On the other hand, the FRET-EM-transfected cells showed a faster decrease in PIP3 levels as just 30 min was time enough to observe a significant decrease, which had been kept up to 90 min (Fig. 3B).

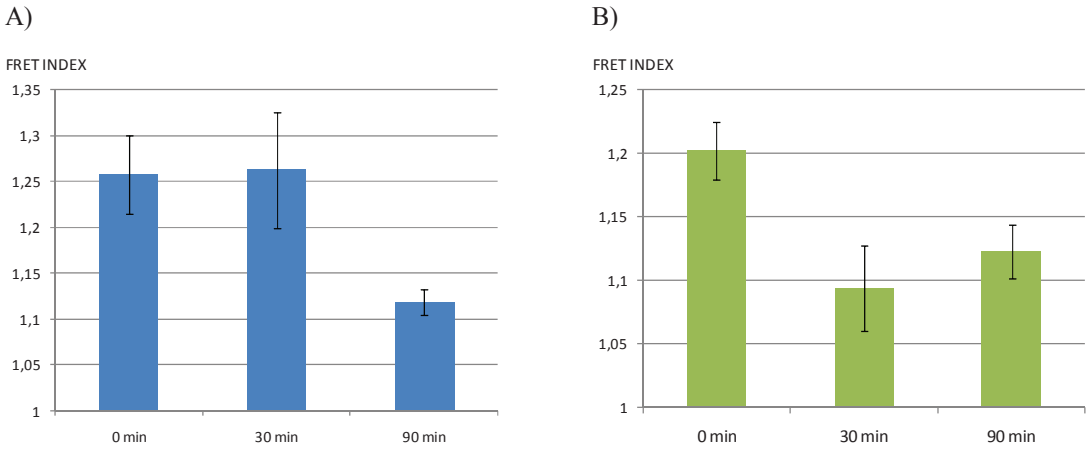

Figure 3. PIP_3 levels in perifosine-treated 4T1 cells. 4T1 cells were transfected with FRET probes for plasma membrane (A) or endomembranes (B) and treated with 10 µM perifosine for 0, 30 or 90 min. Cells were fixed, washed and mounted as described in Materials and Methods. FRET index was calculated and represented, in arbitrary units, for all perifosine exposure time.

Discussion

FRET microscopy is a new technique that has been recently applied for study of phosphoinositides. It provides a very solid method for detecting small variations of PIP3 levels (Sato et al., 2003). As explained above PIP3 production entails AKT activation so that disruption of its levels could well affect AKT activation and thus signaling pathways related with cell survival, proliferation or migration. For this reason PI3K is an interesting target in cancer progression and modulation by APLs. One of the most interesting benefits of FRET is that probes can be oriented towards specific membranes instead of measuring PIP3 levels in

the whole system. Additionally, other phosphoinositides more than PIP3 can me monotorized, giving new information about early steps of the pathway we are focusing.

APLs, and specially perifosine, have been reported to affect PI3K/AKT pathway by reduction of AKT phosphorylation status which entails inhibition of this signaling pathway for cell survival (Sun, 2010; Zitzmann et al., 2012). As phosphorylation of AKT is dependent of PI3K we wondered if perifosine could affect this upstream enzyme. It has been suggested that perifosine inhibits the phosphorylation of AKT as a consequence of an impairment of its translocation to plasma membrane, and subsequent activation (Kondapaka et al., 2003). Aditionally, it has not been described any alteration in PI3K levels nor it activity after perifosine treatment.

In spite of all this reports, our results show that perifosine is decreasing PIP3 levels in 4T1 breast cancer cells remarkably acting in first place in endomembranes and later in plasma membrane. On the other side, FRET microscopy is highly sensitive and more developed than traditional assays. This could be the explanation for the fact that other authors have not detected any alteration in PIP3 levels after treatment with APLs. Despite this however, if this decrease is a consequence of a direct interaction between this APL and the PI3K or, as some studies suggest, could be a metabolic effect of altered lipid profile in biomembranes (Calay et al., 2010; Rios-Marco et al., 2011), remains uncertain. Further studies in this direction will be required for a better understanding of the relation between APLs and PI3K/AKT pathway.

4.7.2. Three dimensional cultures of mammary epithelial cells: perifosine affects acini organization

Introduction

Most of studies aimed at elucidating the action of specific proteins in breast tumorigenesis or identifying inhibitors of proteins that warrant testing in clinical trials have been conducted using the traditional two-dimensional (2D) culture. However, 2D culture does not reflect the important contribution of the tissue microenvironment both in mediation of normal breast tissue viability and in generation of the apoptotic-resistant phenotype of breast tumors (Duong et al., 2012). Despite, culturing of cells in three-dimensional (3D) matrices offers several advantages over 2D culture. 3D systems allows cells to organize into structures that mimic their in vivo architecture, and 3D culture is particularly useful for investigating gene functions and signaling pathways in a more physiologically relevant context. In 3D mammary cultures whereas normal cells become quiescent by day 10 and organize into replicas of human breast acini with correct tissue proportions (Petersen et al., 1992), malignant cells continue to grow, pile up, and form large, disorganized, tumor-like colonies (Muthuswamy et al., 2001). In this sense, mammary epithelial grown in artificial matrix recapitulate numerous features of breast epithelium in vivo, including the formation of acini-like spheroids with a hollow lumen, apico-basal polarization of cells making up these acini, the basal deposition of basement membrane components - collagen IV and laminin V - and, in some cases, the production of milk proteins.

Acini formation in the spontaneously immortalized mammary epithelial human cell line MCF-10A occurs since cells in contact with matrix polarize whereas cells in the center of spheroids lose contact with the extracellular matrix and subsequently undergo apoptosis (Debnath et al., 2002). This three-dimensional cell culture system provides a physiologically appropriate environment for assessing the possible effects of APLs on cell polarization, apoptosis and acini development.

In the present work we set up 3D cultures using an artificial matrix composed primarily of the extracellular components laminin, collagen IV, and entactin (Debnath et al., 2003) and let MCF-10A cells to develop acini (Fig. 1). By using fluorescent microscopy we

show that acini conformation is reached in untreated cells, while perifosine disorganize their structure and induce caspase 3 dependent apoptosis.

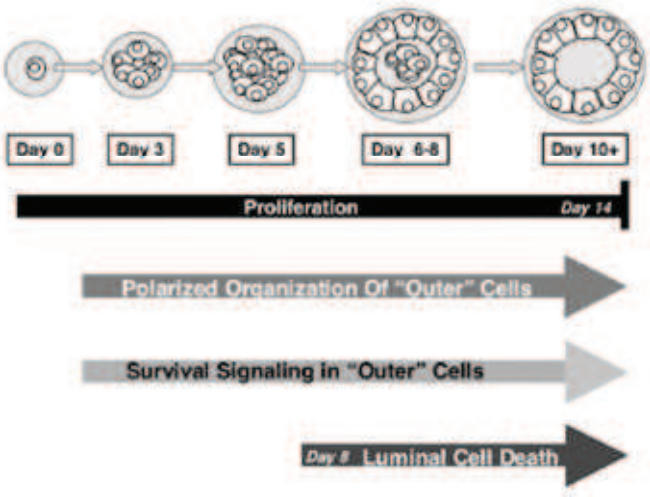

Figure 1. Scheme of biological events during MCF-10A acinar morphogenesis. Acini formation requires cells to be grown in Matrigel and be allowed to grow with some nutrients and growth factor (see Materials and Methods). Cells in contact with proteins of extracellular matrix start to divide and polarize, facing basal membrane towards matrix and apical membrane towards center. Some days later, cells inside the spheroid undergo apoptosis creating the lumen of acini. The period of these processes vary depending on cell type. From Debnath et al., 2003.

Materials and Methods

For acini formation we selected the human breast cell line MCF-10A. These cells grow in monolayer as 2D cultures when seeded in plastic but form acini when cultured over specific artificial matrix. To form acini, cells are usually cultured in a matrix of reconstituted basement membrane derived from Engelbreth-Holm-Swarm tumor (commercially available as Matrigel). Seeding and growth on Matrigel required three different media to be used: Growth, Resuspension and Assay Medium (Table 1).

Monolayer cultures were maintained in Growth medium, kept in confluency until day of the experiment. As Matrigel remains liquid on ice but solidifies rapidly when warmed, we handled it on ice until cover the culture slides. To prepare plates we added 40 µl of Matrigel and spread it using a tip until forming a high meniscus in the border and placed 15 min at 37ºC for it solidification. MCF-10A cells were trypsinized and resuspended in Resuspension

Medium, centrifuged at 1500 rpm for 3 min and pellet was resuspended in Assay Medium. We prepared a stock of Assay Medium + 4% Matrigel + 10ng/ml Epidermal Growth Factor (EGF) and perifosine 10 µM was added to half of it. Both media, with or without perifosine, were mixed 1:1 with cell suspension (5 µM perifosine final concentration). 400 µL of mixtures were plated per well, over solidified Matrigel. Cells were allowed to grow in incubator. Each 3 days perifosine was renewed; medium replacement with Assay medium containing 2% Matrigel + 5 ng/mL EGF was done each 4 days. Pictures were taken in inverted microscope at days 1, 6 and 12. The size of acini structure was measured in control and treatments for all periods using Image J software.

Component	**Growth Medium[a]**	**Resuspension Medium[a]**	**Assay Medium[a] (without EGF)**
DMEM/F12	500.0 mL	400.0 mL	500.0 mL
Horse serum	25.0 mL	100.0 mL	10.00 mL
Epidermal Growth Factor (EGF)	100 ng/mL	—	—
Hydrocortisone	0.5 µg/mL	—	0.5 µg/mL
Cholera toxin	100 ng/mL	—	100 ng/mL
Insulin	10 µg/mL	—	10 µg/mL
Pen/Strep	5.0 mL	5.0 mL	5.0 mL

Table 1. Media recipes for MCF-10A cells 3D cultures. For each medium type, premix all of the appropriate additives, sterile filter through a 0.2-µm filer, and add to DMEM/F12 medium bottle. From Debnath et al., 2003.

In order to check polarization markers, the cells had to be fixed and immunostained. Different buffers for immunostaining were prepared as indicated in table 2. Fixation was done with methanol:acetone (1:1) at 20ºC for 15 min. Cultures were permeabilized using 0.5% Triton X-100 in PBS for 10 min at 4º C and rinse done three times with PBS/Glycine 10–15 min per wash at room temperature. The block was done in IF Blocking Buffer for 2 h at room temperature. We incubated with (1:100) anti cleaved-caspase 3 primary antibody in IF blocking Buffer at 4ºC overnight. Rinses were done three times (20 min each) with IF Buffer at room temperature with soft rocking. For secondary antibody incubation we used rhodamine secondary conjugated antibody against anti-cleaved-caspase 3 primary antibody and a second

staining consisting in conjugated FITC fluorescent primary antibody against Laminin V (1:200), all in IF Blocking Buffer for 40–50 min at room temperature. Samples were rinsed three times (20 min each) with IF Buffer at room temperature with gentle rocking. To counterstain nuclei, 0.5 ng/mL 4,6-diamidino-2-phenylindole (DAPI) was added for 15 min at room temperature. For mounting, Mowiol was added between Matrigel and coverslip and both parts were stuck very kindly. Samples were analysed by confocal microscopy.

	PBS/Glycine	IF Buffer	IF Incubation Buffer
NaCl	130 mM	130 mM	130 mM
Na_2HPO_4	7 mM	7 mM	7 mM
NaH_2PO4	3.5 mM	3.5 mM	3.5 mM
Glycine	100 mM	100 mM	100 mM
NaN_3	—	7.7 mM	7.7 mM
Bovine serum albumin	—	0.1 %	0.1 %
Triton X-100	—	0.2 %	0.2 %
Tween-20	—	0.05 %	0.05 %
Goat serum	—	—	10 %

Table 2. Buffers for immunofluorescence of acini.

Results

MCF-10A cells started to grow from the first day after being seeded in Matrigel. Instead of spreading along the surface, as they do in monolayer cultures, they started to divide but kept together, forming rounded spheroids. These cell masses were bigger on day 6 and 12 (Fig. 2A). Regarding the acini shape, we could find some differences between control and perifosine-treated cells. At the beginning, the acini development was similar in both situations, but some altered acini could be observed on last days of perifosine treatment (squares of figure 2). Control samples of 12^{th} day showed mainly a well round shape acini with higher cell density on periphery, suggesting a partial emptiness inside them (Fig. 2B). However, in perifosine treatment several acini were shape altered, characterized by unrounded

and disaggregated structures (Fig. 2C). The "healthy" acini present in perifosine treatment did not have any size variation compared to control during all the development process (Fig. 2D).

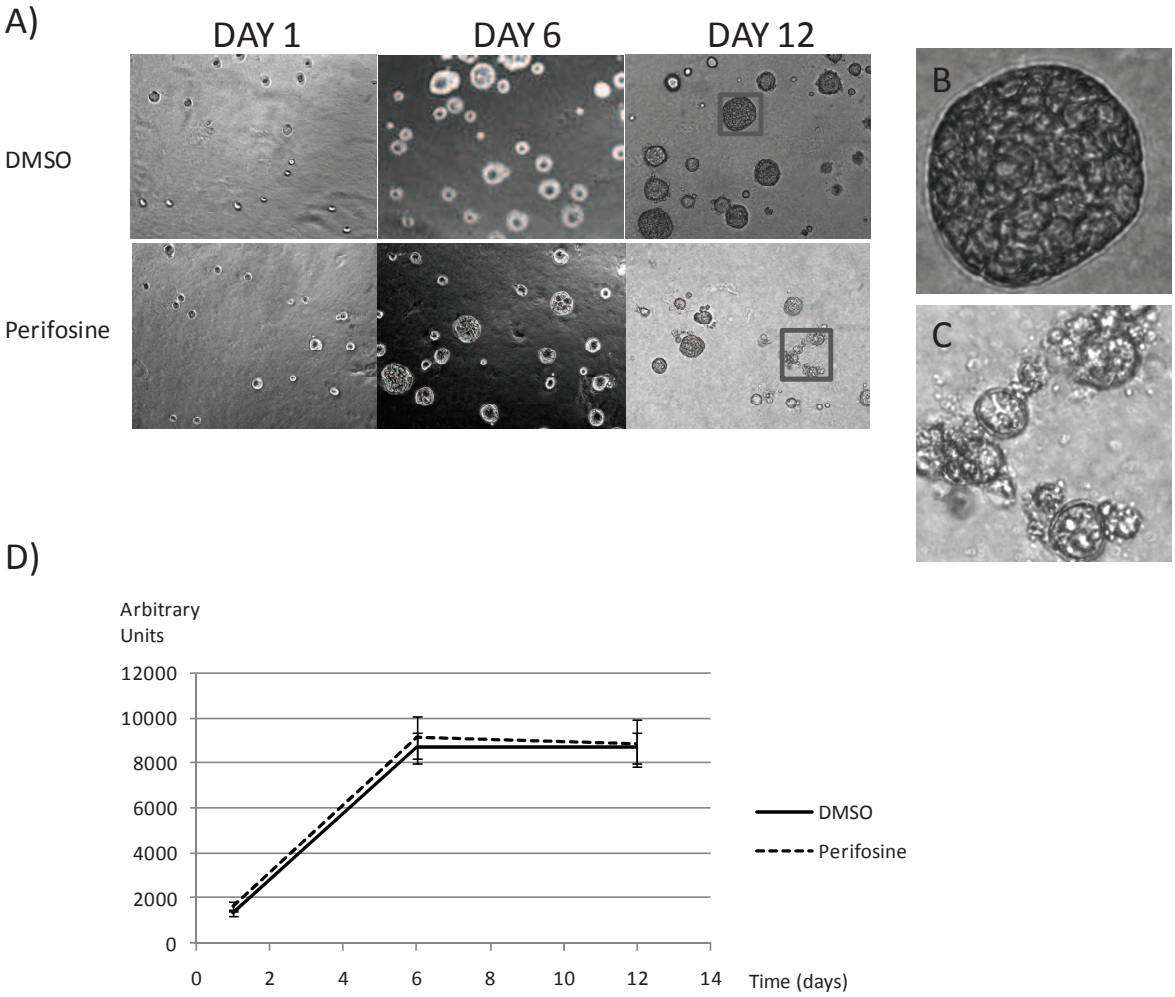

Figure 2. Acini development of mammary epithelial human cells on Matrigel. MCF-10A were seeded on Matrigel as explained in Materials and Methods and DMSO (control) or 5 μM perifosine was added in Assay Medium containing 2% Matrigel + 5 ng/mL EGF at day 0. Acini were allowed to develop until day 12. During this time, medium and perifosine were renewed each 3-4 days. Contrast phase pictures were taken at day 1, 6 and 12 (A). Most of the acini were well developed in control (B), while some disaggregated structures could be found in perifosine-treated cultures (C). Unaltered acini in control and treated cultures were selected and its diameter was measured using image J software for 50-100 acini/treatment and represented (D).

Immunofluorescence of polarization markers allowed us to check acini morphology and their development. Confocal microscopy in equatorial cross sections of control acini showed that cell nuclei (DAPI staining) are disposed as a ring around a hollow lumen. These structures were patent from days 4-6 in controls. Some active caspase 3 staining could be found confirming the necessary cell death inside developing acini for lumen formation. Laminin 5, a basal membrane marker of polarized cells, was present in acini evolving peripherically the whole system (Fig. 3). Interestingly, in perifosine-treated cultures, some acini resulted to be incomplete, with a lack of Laminin 5 at some points of the rounded structure (Fig. 3, arrows). In these areas DAPI staining was lower too and caspase 3 was activated. All this indicates that some perifosine-treated acini were disorganized, being unable to develop properly.

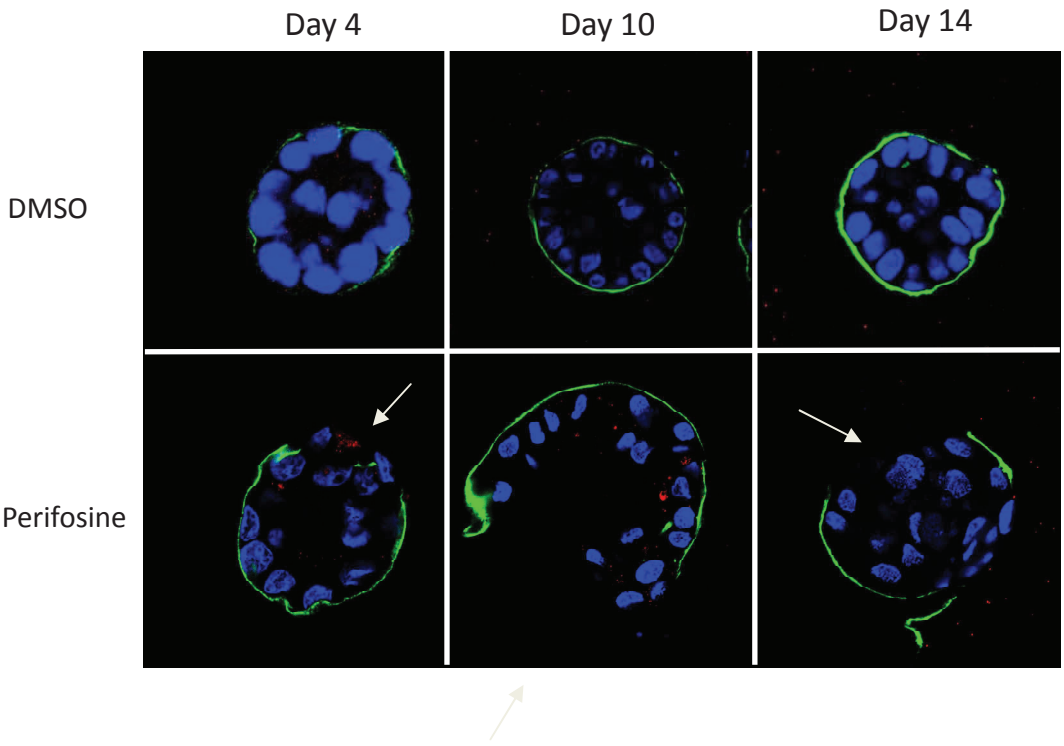

Figure 3. Immunostaining of MCF-10A acini. MCF-10A were seeded on Matrigel as explained above and DMSO (control) or 5 µM perifosine was added in Assay medium containing 2% Matrigel + 5 ng/mL EGF at day 0. Acini were allowed to develop until day 14. During this time medium and perifosine were renewed each 3-4 days. Slides were fixed and immunostained as explained in Material and Methods at days 4, 10 and 14. Samples were visualized by confocal microscope for DAPI (blue), Laminin V (green) and cleaved-caspase 3 (red). In control, nuclei have a radial disposition surrounded by Laminin V while perifosine show interrupted Laminin V staining pattern (arrows).

To check apoptosis some pictures were taken too at lower magnification and with different exposure times. We could see that treatment not only disorganize acini but also any increase in active caspase 3 staining compared to control cells (Fig. 4). All this data suggest that perifosine is impeding correct acini formation, causing a disorganization of structures and promoting caspase 3 dependent apoptosis in mammary epithelial cells MCF-10A.

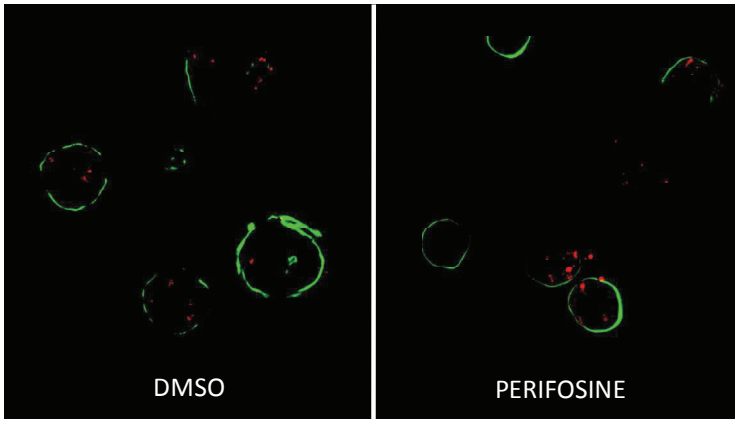

Figure 4. Activation of cleaved caspase 3 by perifosine treatment. MCF-10A were seeded on Matrigel as explained above and DMSO (control) or 5 μM perifosine was added in Assay medium containing 2% Matrigel + 5 ng/mL EGF at day 0. Acini were allowed to develop until day 10. During this time medium and perifosine were renewed each 3-4 days. Slides were fixed and inmmunostained as explained in Materials and Methods at days 10. Samples were visualized by confocal microscope for Laminin V (green) and cleaved caspase 3 (red). Perifosine-treated cultured acini showed higher caspase intensity than control ones.

Discussion

Our results show how mammary epithelial human MCF-10A cells grow and develop acini 3D structures when they are cultured in artificial matrix. The acini development is a complex process that requires cells to become polarized, facing it basal membrane towards extracellular matrix and the apical face to lumen. To achieve the acini morphology it is necessary that cells inside the spheroids suffer cellular death; since they are not in contact with matrix and they do not grow, they initiates apoptotic cellular death (Tang et al., 2011).

There exist no reports at all about acini cultures and APLs making difficult to evaluate the effect of perifosine on acini development. Some authors have reported in acini that the cells in contact with matrix have a high PI3K/AKT activity (Debnath et al., 2002). This

suggest that perifosine, which has been reported by us and others as PI3K/AKT pathway inhibitor (Rios-Marco et al., 2011; Zitzmann et al., 2012), could have a more marked effect in these cells causing a disorganization in acini´s periphery, instead of affecting the whole system. On regards the polarization, Laminin V is present both in perifosine-treated and control acini. This is a probe that there is a basal membrane in cells forming the acini, so the polarization itself seems not to be altered by perifosine.

It is interesting to remark that, although apoptosis is not the only cell death suggested in lumen of acini (Debnath et al., 2002), this is an essential process for its development. Scarce apoptosis could be a consequence of overexpression of pro-oncogenes and indeed a malignant cancer phenotype with no lumen inside the spheroids has been reported in transformed MCF-10CA1 cell line, which is fully malignant (Imbalzano et al., 2009). On the other hand too much apoptosis could entail the death of the whole system, impeding breast acini to develop properly. In our study we have detected a slight increase in cleavage of caspase 3 of perifosine-treated acini when compared to controls. It is still not clear however whether this increase is a proapoptotic direct effect of the perifosine or a posterior result of the alteration in periphery of acini caused by the agent. More studies with APLs should be done in 3D models for understanding the way these compounds could affect the tridimensional status and development of breast tissues.

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Discussion

5. Discussion

Beating the cancer is one of the most important aims for researchers that work in biomedicine. This illness is spread all around the world and few are the effective treatments that could ensure a complete healing or an absence of secondary effects. APLs are actually used in clinical trials against mammary or renal carcinomas (Clive et al., 1999; Cho et al., 2012) as well as tested in other tumoral cell lines (Rybczynska et al., 2001). Since the exact mechanism by which APLs fight the cancer is not completely know, the main aim of this thesis work has been to study their antiproliferative activity and the mechanisms underlying its biological action. We have focused it towards the effect of APLs on cholesterol homeostasis and some other lipid dependent processes that we found to be altered by them.

One of the preliminary and compulsory ways to evaluate APLs cytostatic activity was performing cell growth studies in presence of the tested agents. APLs have shown to clearly decrease proliferation of cancer cells in a dose dependent manner; their antiproliferative potency activity is different in HepG2 and U-87 MG cells, being IC_{50} noticeably lower in the glioblastoma cell line, and thus more effective. However, a similar pattern of antiproliferation has been found in both types of cells, edelfosine being the agent with the highest activity while HePC the lowest one. It is clear that antitumor activity of APLs differs depending on the cell type or APL in question (van Blitterswijk and Verjeij, 2008). Although none study has simultaneously evaluated the antiproliferative activity of the four APLs, some authors have compared APLs inhibitory effects in other cell lines obtaining similar results. For example, Wieder et al. (1995) reported in MDCK cells that edelfosine and HePC inhibit cell proliferation with IC_{50} of 75 and 135 μM respectively.

By measuring lactate deshidrogenase released to medium we were able to see in which degree APLs would produce cell lysis, instead of growth inhibition. On this regard, high-doses for short-time treatment resulted not cytotoxic in HepG2 (e.g. 100 μM HePC for 6 h) and neither were smaller amounts after one day treatment (e.g. up to 75 μM in HePG2 cells or up to 50 μM in U-87 MG cells). This suggests that the decrease in cell number, quantified by cristal violet staining, after APL-treatment is mostly due to antiproliferative but not cytolitic activity.

Antiproliferation is clearly related with cell cycle progression. It has been previously reported that there exist cell growth arrest caused by APLs in some cells lines (Celeghini et al., 2011). Control points in cell cycle are located at G1, G2 and M; from results of chapter 4.4. we deduce that APLs promote cell cycle arrest in U-87 MG cells at some point within phases G2/M. G2 arrest point has been related with DNA-damage dependent signaling (van Vugt and Yaffe, 2010). Since APLs are not reported to damage DNA it would reasonable to think that cancer cells treated with them might be able to overcome the DNA-damaged dependent G2 arrest point. Moreover, one of the proteins involved in the G2 checkpoint is cdc2 kinase, which activation requires its dephosphorylation at Thr14 and Tyr15 residues (Sánchez and Dylancht, 2005). In this work we have found out that cdc2 is in fact active after APL-treatment, which suggests that the arrest caused by APLs should be located at some point of mitosis, as occurs with other anticancer drugs as vinblastine (Singh et al., 2008; Rai et al., 2012).

In our study, not only has the cell cycle been demonstrated to be altered by APLs as part of their anticancer activity. Electron microscopy allowed us to deep on changes in morphology caused by APLs and even more, to detect if some metabolic processes could be affected too. In pictures exposed in chapter 4.4. it is shown that APLs-treated glioblastoma cells are characterized by abundant autophagic vacuoles and autolysosomes, which are consistent with autophagy (Bursch et al., 2000; Komata et al., 2000). Autophagy is a highly conserved process that is actually in discussion since some authors suggest that it has a protective effect for the cell (Kaushal, 2012) while some others link it to apoptosis and cell death (Das et al., 2012). There are almost no reports to date that relate autophagy with APLs and so, it is not clear why this process is happening in APL-treated U-87 MG cells. Recently nevertheless, Shi-Yong Sun has reported in a mini-review (2010) that the proapoptotic action of perifosine over cancer cells is enhanced when autophagy is inhibited with drugs and that this effect is clearly dependent on PI3K/AKT/mTOR signaling pathway. On balance, although this question still remains uncertain, enhanced autophagy could be a protective mechanism of U-87 MG cells against APL-antiproliferative action.

Several studies conclude that cholesterol metabolism is essential in cell processes such as proliferation and differentiation and because of this, it has been highly imperative for us to study the action of APLs on cholesterol homeostasis in tumoral cells. As explained in

Introduction, APLs are thought to act on cell membranes. In this sense, Barrat et al. (2009) have done some experiments exposing cells to raising concentrations of HePC. The point is made when they suggest that if concentration of this APL is higher than critical micellar concentration (CMC), it forms extracellular micelles and is then inserted into membranes as monomers groups. After that, the APL is thought to cross the membrane and possibly released towards cell inside where it would exert its biological action. It is very reasonable therefore that, because of their phospholipid-like structure, APLs could interact with membrane lipids like cholesterol and alter their location and homeostasis.

The cholesterol homeostasis needs of an accurate and balanced transport system between outside and inside of the cell, and from the different membranes. The dynamism of the cholesterol trafficking has made very difficult to study which proteins or organelles are exactly involved on it. It is clear however that part of the cholesterol that is internalized must reach the ER – where cholesterol sensing mechanisms are located – and be stored through its esterification.

In the first work of this thesis we analyze in detail if HePC affects at any point the cholesterol trafficking inside HepG2 cells. Radiolabeling of newly synthesized cholesterol, which is supposed to quickly reach plasma membrane (Chang et al., 2006), and posterior extraction demonstrated that transport of cholesterol from ER to plasma membrane was not affected by this APL in HepG2 cells. However labeling of external cholesterol belonging to LDL particles showed that there is a significant decrease in the amount of radiolabeled cholesteryl esters in cells treated with HePC, which indicates that there exists at some point an inhibition of cholesterol internalization by LDLR and consequent transport to the ER.

Some time-course studies from Cruz and Chang (2000) showed that labeled LDL-cholesterol incubated in CHO cell cultures takes approximately 30 min to reach the plasma membrane, whereas 60-90 min are required to it become esterified at the ER; this together suggests that cholesterol internalized reaches first plasma membrane and from there it travels to the ER. In our case, by short incubation of HePG2 cells with radiolabeled LDL-cholesterol and posterior use of MβCD, we detected how much cholesterol from lipoproteins reached plasma membrane in HePC-treated cells (chapter 4.1.). Interestingly, we found no differences in cholesterol extracted with cyclodextrines between control and HePC-treated cells. This indicates that cholesterol transport is not inhibited from lipoprotein internalization up to

plasma membrane, and thus the inhibition in transport caused by HePC must be located between plasma membrane and ER. In addition, by direct plasma membrane´s cholesterol radiolabeling, we have extensively confirmed this traffic disruption with all APLs (see chapters 4.2., 4.4., 4.5. and 4.6.). So summarizing, we have discovered for the first time that all APLs share a common direct effect on cholesterol homeostasis that is to disrupt its transport from the plasma membrane to the ER.

Such transport is thought to follow two routes: 1) vesicular route via endosomes -ATP dependent - and 2) non vesicular alternative route. In our first work we report in cells exposed to HePC that the cholesterol transport is inhibited in either presence or absence of ATP which suggests that the non vesicular route is the one affected by APLs. Furthermore, LDL-cholesterol uptake is known to be mediated by endocytic/lysosomal compartment. In this process there are two proteins involved that are located into LE/LY, these are NPC1 and NPC2. It has been described that when any of these proteins are mutated (e.g. NPC disease) there is an accumulation of lipids inside these organelles; this phenotype can be detected by microscopy as a filipin staining inside cells in a vesicular pattern which colocalize with LE/LY markers as LAMP1 or LAMP 2 (Reiners et al., 2011; Appelqvist 2011). Moreover, there exist some drugs such as U18666A which interfere with cholesterol trafficking and thus mimics NPC phenotype (Mohammadi et al., 2001). What is more, cells treated with this compound have significantly lower cholesterol esterification and higher biosynthesis (Lange, 1994; Issandou et al., 2004). In fluorescent microscopy studies from chapter 4.4. we conclude that in U18666A-treated U-87 MG cells the cholesterol is accumulated into LE/LY compartment. Surprisingly, however, these glioblastoma cells do not present this fenotype when are exposed to APLs. This, together with the data from the studies of cholesterol transport in ATP-free conditions, states clearly that APLs alter non vesicular cholesterol transport from the plasma membrane to the ER; as opposite as U18666A, which accumulate lipids inside vesicular endosomal compartments.

An additional fact that has been probed regarding cholesterol trafficking is that the differential membrane-domains labeling is achievable. Firstly, it is proposed that plasma membrane cholesterol exists in two states that differ on the membrane fluidity. While cholesterol present in L_d domains (non-raft) has higher tendency to escape, the cholesterol from L_o domains (raft) is thought to be more retained inside the membrane (Lange and Steck,

2008). So, it is reasonable to think that a short cholesterol labeling would just label non raft regions whereas an extended labeling period would label both raft and non-raft (e.g. in HepG2 cells, raft regions were radiolabeled for 15 min while the whole membrane labeling spent 60 min). Secondly, sphingomyelinase-treatment can be used to increase cholesterol transport from plasma membrane to ER because it increase cholesterol/sphingomyelin ratio above its threshold level so that cholesterol is increasingly internalized for its storage. Additionally, it is known that sphingomyelinase hydrolyzes the sphingomyelin mostly in raft and consequently promote cholesterol release from these domains (Chatterjee, 1994; Abi-Mosleh et al., 2009). All this in mind, the differential labeling study from chapter 4.6. points out interesting results. In HepG2 cells the sphingomyelinase increase cholesterol esterification after long-time labeling, but remarkably not after short-time´s. This confirms that cholesterol incorporates firstly to the non-raft regions and later to the raft domains. However, when differentially labeled cells were exposed to HePC we found out that cholesterol esterification was reduced in either case. In conclusion, HePC inhibits the cholesterol transport that comes from both raft and non-raft regions while the sphingomyelinase enhance its internalization from mainly rafts (Fig. 1); this suggests that APLs affect the whole membrane and its effect cannot be avoided with a cholesterol transport enhancement by lipid modulators as sphingomyelinase.

Since the cholesterol sensing mechanisms are mainly located at the ER, the trafficking of cholesterol is expected to be essential for a proper regulation. Cholesterol content in this organelle is really poor which means that the minimum alteration on its transport, that will affect its arriving to ER, is accurately detected so that homeostatic response would be no longer delayed.

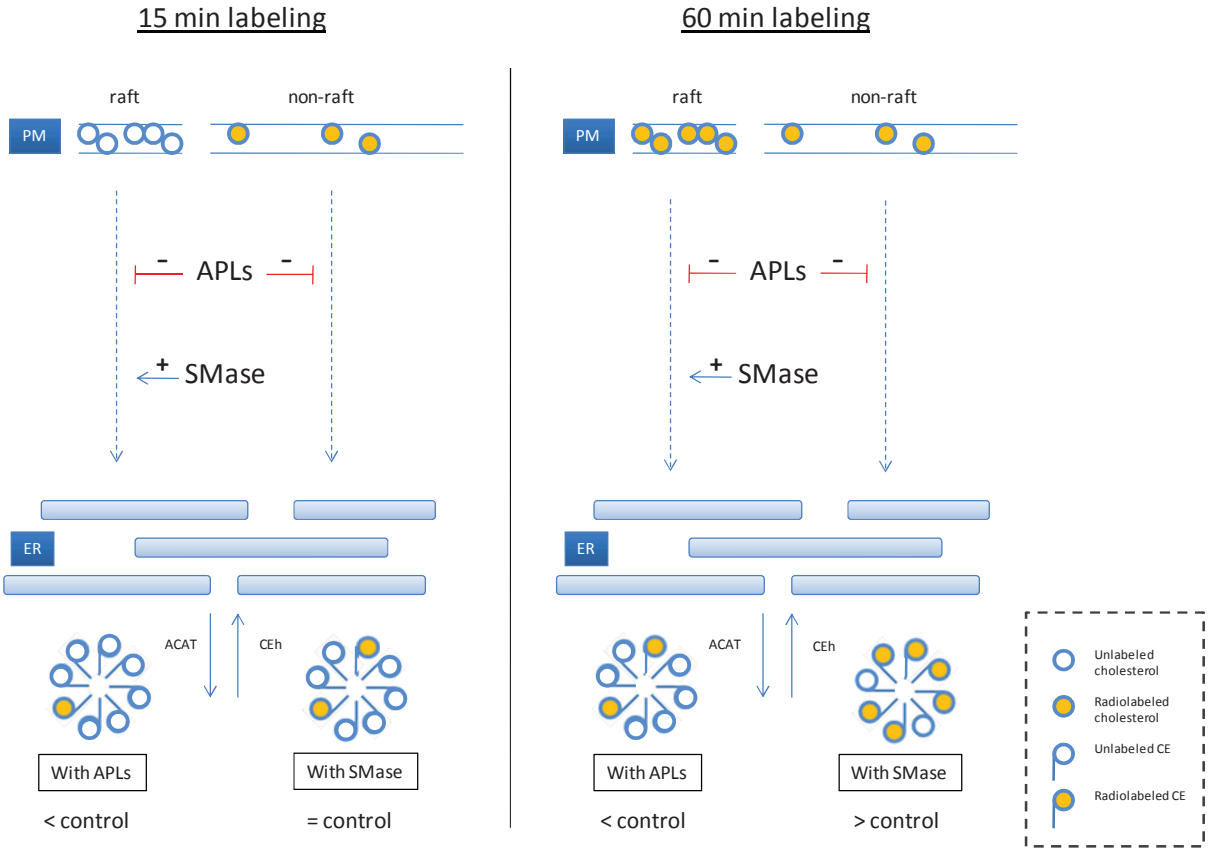

Figure 1. Differential labeling of cholesterol in plasma membrane. Cells labeled with [^{3}H-cholesterol] incorporate first this cholesterol to non-raft regions (left), while longer exposure-time labels both raft and non-raft domains (right). No radioactivity is obtained in cholesteryl ester fraction in just non-raft labeled cells in presence of sphingomyelinase (SMase). On contrary, when raft and non-rafts fractions are labeled, SMase treatment does promote radioactive cholesteryl ester formation. APLs decreased cholesterol esterification in either case, indicating a disruption of cholesterol coming from both raft and non-raft domains. Abbreviations: acyl-CoA:cholesterol acyltransferase (ACAT), cholesteryl ester hydrolase (CEh).

Previous studies have described that mammalian cells produce not only cholesterol but also high amounts of its metabolic precursors (Echevarria et al., 1990; Lange et al., 1991). In the cycle of cholesterol synthesis these precursors leave ER, rapidly reach plasma membrane and go back to ER to complete cholesterol synthesis (Heino et al., 2000; Field et al., 2007). The results from chapter 4.1. indicate that HePC increase some intermediates of cholesterol synthesis as lathosterol or desmosterol and in addition, their traffic seem to be partially impeded before they reach back ER, as occurs with cholesterol itself. Moreover, we have demonstrate that APLs increase the incorporation of radiolabeled acetate to cholesterol in

HepG2 cells as well as in U-87 MG. All this clearly demonstrates that, the de novo synthesis of cholesterol is increased after APL-treatment, and so it is highly accumulated in tumoral cells. Furthermore, main regulatory enzymes of biosynthetic pathway as HMGCR, FDPS and FDFT1 are induced too by APLs. All this together represents a conclusive evidence of an altered feedback regulation of cholesterol in cancer cells treated with APLs; which are not able to properly detect the intracellular levels of cholesterol and therefore, they keep synthesizing and accumulating it.

Cholesterol biosynthesis is not the only homeostatic response that has been probed to be affected by APLs. Cholesterol catabolism and related pathways as bile acid or steroid hormones synthesis are essential for cholesterol homeostasis. Part of the work of the thesis has been dedicated to check the gene expression profile regarding cholesterol anabolism, trafficking, catabolism and efflux. However, lipids are not genetically encoded and so genome or transcriptome can only reflect cholesterol homeostasis indirectly (Ikonen, 2008). In addition, many of the proteins that preserve cellular cholesterol homeostasis exhibit considerable functional redundancy, apparently to benefit of the organism but to frustration of the researcher.

Nevertheless, it has been demonstrated by us that APLs increase the active isoform of SREBP-2 which travels to nucleus and stimulate some target lipid-related genes. Interestingly, APL-treatment upregulated the gene expression and enhanced the protein levels of LDLR and HMGCR that are well known target genes of SREBP-2 (Brown and Goldstein, 2009). This is coincident with the results from metabolic assays mentioned above and thus strongly indicates that cholesterol biosynthesis and uptake are upregulated by APLs as a consequence of the defective cholesterol arrival to ER.

Another genes, related with cholesterol trafficking and regulation, are broadly studied in chapter 4.3., being noteworthy some of the next. Niemann Pick C1-like 1 (NPC1L1) contributes to internalization of cholesterol in liver and is modulated by its cholesterol content (Betters and Yu, 2010). This protein is supposed to participate in cholesterol uptake and is upregulated by APLs. NPC1L1 modulates as well bile acid concentration; indeed it overexpression has been found to reduce the bile acid concentration up to 20 times (Temel et al., 2007). On the other side, treatment with APLs downregulates low-density lipoprotein receptor-related protein associated protein (LRPAP1), located in ER, which seems to be

involved in cholesterol clearance (Pandey et al., 2008). Additionally Insig 2, which is essential in cholesterol feedback regulation, has been found to be downregulated too so that the complex SREBP-Scap is expected to be translocated and thus activated; interestingly this fits with SREBP-2 activation mentioned above.

Regarding the tissue-linked lipid catabolism, hepatic cells are specialized in the specific route of bile acid synthesis. Primary bile acids – in human chenodeoxycholate and cholate – are the end products of cholesterol catabolism, which are conjugated in the liver to taurine or glycine to increase their water solubility. Nascent bile formation occurs at the level of the hepatic canalicular apical membrane, driven by active secretion of bile acids by the energy-dependent ABC transporter ABCB11 (Gerloff et al., 1998). The other two major lipids contained in human bile are cholesterol and PtdCho whose secretion is catalized by other ABC transporters (Gadaleta et al., 2010). In bile acid formation, cholesterol is converted into cholic acid by two routes: classic pathway and alternative pathway. We found out by PCR analysis that 7α-hidroxylases CYP7A1, CYP7B1 and CYP39A1, which participate in either of these pathways, are transcriptionally downregulated by APLs. It is reasonable to think that if oxysterol production is decreased by APLs, the activation of LXR – one of the oxysterols target transcription factors – will be inhibited too. This could well explain why some downstream target genes of LXR such as ABCA1, apoE, or SREBP-1c (Zhao and Dahlman-Wright, 2010; Faulds et al., 2010) are found to be repressed by APLs in the gene expression profile carried out in chapter 4.3..

In the matter of cholesterol efflux we have reached interesting results. Some of the main participants in cholesterol efflux are the ABC transporters. ABCA1 for example, is essential in cholesterol and phospholipids transport towards extracellular aceptors since it yields these lipids to apoA1 for HDL formation (Cavelier et al., 2006). Data from our work in chapter 4.3. shows that in HepG2 cells APLs promote not only downregulation in ABCA1 gene but decrease the cholesterol efflux to ApoA1. Regarding the reverse cholesterol transport context, it is interesting to remark that hepatoma cells have a lower gene expression for proteins involved in formation of HDL, as CETP and LCAT. All this suggests that treatment with APLs promotes downregulation of genes involved in cholesterol efflux, probably as consequence of impairment in cholesterol sensing caused by these agents.

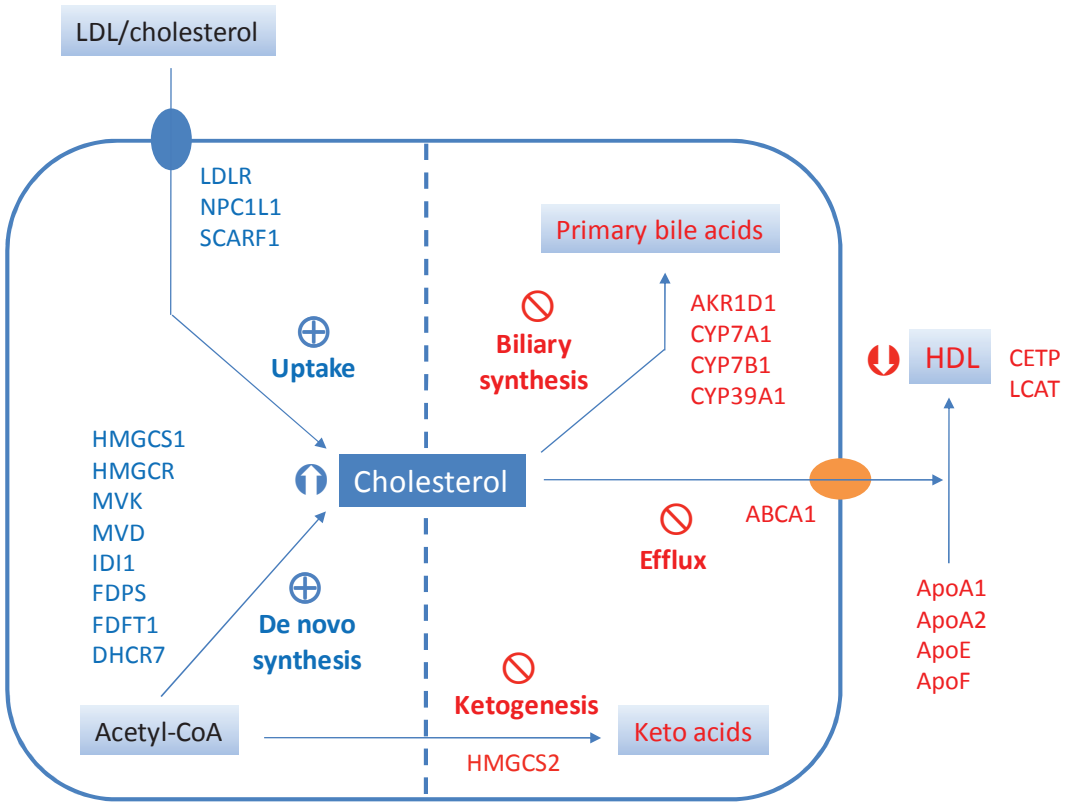

Figure 2. Effect of alkylphospholipids on genes involved in cholesterol homeostasis in HepG2 cells. APLs upregulate genes related with de novo synthesis and uptake of cholesterol and downregulated catabolism, efflux and reverse cholesterol transport involved genes. Abbreviations used: ABCA1 (ATP-binding cassette, sub-family A (ABC1), member 1), AKR1D1 [aldo-keto reductase family 1, member D1 (delta 4-3-ketosteroid-5-beta-reductase)], APOA1 (apolipoprotein A-I), APOA2 (apolipoprotein A-II), APOE (apolipoprotein E), APOF (apolipoprotein F); CETP (cholesteryl ester transfer protein, plasma), CYP7A1 (cytochrome P450, family 7, subfamily A, polypeptide 1), CYP7B (cytochrome P450, family 7, subfamily B, polypeptide 1), CYP39A1 (cytochrome P450, family 39, subfamily A, polypeptide 1), DHCR7 (7-dehydrocholesterol reductase), FDFT1 [farnesyl-diphosphate farnesyltransferase 1 (squalene synthase)], FDPS [farnesyl diphosphate synthase (farnesyl pyrophosphate synthetase, dimethylallyltranstransferase, 22 geranyltranstransferase)], HMGCR (3-hydroxy-3-methylglutaryl-coenzyme A reductase), HMGCS1 [3-hydroxy-3-methylglutaryl-coenzyme A synthase 1 (soluble)], IDI1 (isopentenyl-diphosphate delta isomerase 1), LCAT (lecithincholesterol acyltransferase), LDLR (low-density lipoprotein receptor), MVD (mevalonate (diphospho) decarboxylase), MVK (mevalonate kinase), NPC1L1 [NPC1 (Niemann-Pick disease, type C1, gene)-like 1], SCARF1 (scavenger receptor class F, member 1).

Summarizing, transciptome studies have shown that APLs repress processes that will reduce cholesterol levels inside the cell as its catabolism, for bile acid formation, or its efflux. At the same time however, synthesis and uptake of cholesterol are clearly increased as part of a feedback response. All this indicate that hepatoma cells suffer a dramatic misregulation of

cholesterol sensing mechanism, probably due to the impairment of cholesterol arrival to ER; the consequence is that cell accumulates cholesterol as if there were almost no availability of it in a useless try of the cell to keep the normal sterol homeostasis (Fig. 2).

Membrane domains are acquiring increasingly importance as there is growing evidence that links them with signaling pathways. Raft microdomains have been in discussion in the last decade, especially because of the difficulty in their isolation and analysis. It is reasonable to think that cholesterol homeostasis alteration may as well affect lipid composition in plasma membrane and specifically in structural domains such as raft. In this sense Sanchez-Wandelmer et al. (2009) reported that when cholesterol biosynthesis is disrupted raft structure and function is altered too. There is a crucial point in methodology used for the raft isolation, for this reason we performed a free-detergent isolation method which allowed us to determinate not only protein but also lipid content on them (Macdonald and Pike, 2005). Interestingly, HePC increase the cholesterol/sphingomyelin ratio in raft fractions in HepG2 cells and so, we hypothesized that this might destabilize raft domains. On this regard, it is demonstrated that the depletion of cholesterol by cyclodextrins results in raft disruption and malfunction of numerous signal transduction pathways (Gajate et al., 2009; Park et al., 2009); in addition, enrichment of the membrane with cholesterol also destabilizes membrane raft (Matkó and Szöllösi, 2005). This in mind, cholesterol alteration caused by APLs is expected to destabilize raft and their dependent signaling pathways.

As explained under Introduction, the PI3K/AKT pathway is a well described route that seems to be overactivated in cancer cells; it is thought to be dependent of raft stability and cholesterol content. Some APLs, especially perifosine, have been reported to inhibit this route mainly at level of AKT (Kondapaka et al., 2003; Lucas et al., 2010). In results of chapter 4.5. we describe how perifosine and edelfosine, when acting as cholesterol depleting agents, as well as MβCD, inhibit the AKT phosphorylation. Interestingly, when cholesterol is repleted after MβCD-treatment the P-AKT levels were increased back to normal. However, replenishment of cholesterol after APL-treatment was not complete and neither the recovery of AKT phosphorylation was. This is strongly indicative that there is a relationship between AKT inactivation and cholesterol depletion caused by APLs.

Nevertheless, it still remains unclear if the inhibition of AKT is a direct effect of APL on the kinase and/or a result of the cholesterol disruption. One interesting contribution to this

riddle is the FRET study that we carried out in collaboration with Dr. Bart Vanhaesebroeck. We have observed for the first time that PIP3 levels inside 4T1 breast cancer cells were modulated by perifosine. This agent caused a reduction in the phosphoinositide levels, in first place in endomembranes and later in plasma membrane; that might not have been detected by other methods. This effect is in consonance with the inhibition of phosphorylation of AKT and, although more experiments should be done in this direction; this could help in the understanding of the interaction between APLs and PI3K/AKT pathway.

Since cholesterol homeostasis is determined by the balance of cholesterol uptake, trafficking and efflux we have also investigated the effect of APLs on release of cellular cholesterol when these agents are added in serum-free medium. As opposite to the results regarding ABC transporters that are mentioned above, experiments from chapter 4.5. show that APLs highly promote cholesterol efflux from HepG2 cells to extracellular medium in absence of serum. In fact, our laboratoty has reported for the first time that APLs can act as extracellular cholesterol acceptors. The kinetic of cholesterol sorting is bi-exponential, with a fast sorting up to 5 min that is stabilized later and reach its maximum level around 30-45 min. The kinetic could match with two different cholesterol pools inside the plasma membrane, with a fast release lasting a few minutes followed by a slower efflux thereafter (Zidovetzki and Leviatan, 2007). Interestingly, APLs promoted cholesterol sorting in a dose dependent manner, suggesting a direct interaction of APL with the sterol. ErPC however is an exception, as we found no differences in cholesterol efflux ability between the assayed concentrations of this agent (from 10 to 50 μM).

Moreover, the capability of APLs to promote cholesterol efflux is modulated by the presence of cholesterol or lipid acceptors in the medium. When cholesterol is coadded to APL the efflux is partially impeded, with the exception of ErPC. In addition, when we exposed HepG2 cells to HePC in serum-containing solutions the cholesterol efflux was completely abolished. As it has been published that APLs are able to form extracellular micelles (Barrat et al., 2009), we hypothesize that in serum-free media APLs form micelles that are able to accept cellular cholesterol; this ability however is reduced by the presence of extracellular cholesterol acceptors, or cholesterol itself, thus decreasing APL´s effective concentration (Ménez et al., 2007). It is interesting to remark that the differences between ErPC and the other APLs could be explained by its singular double bond within its structure that would

promote lamellar instead micellar monomers association (Dymond et al., 2008). In the Figure 3 we show a possible model of interaction of APLs with cholesterol in relation with its efflux.

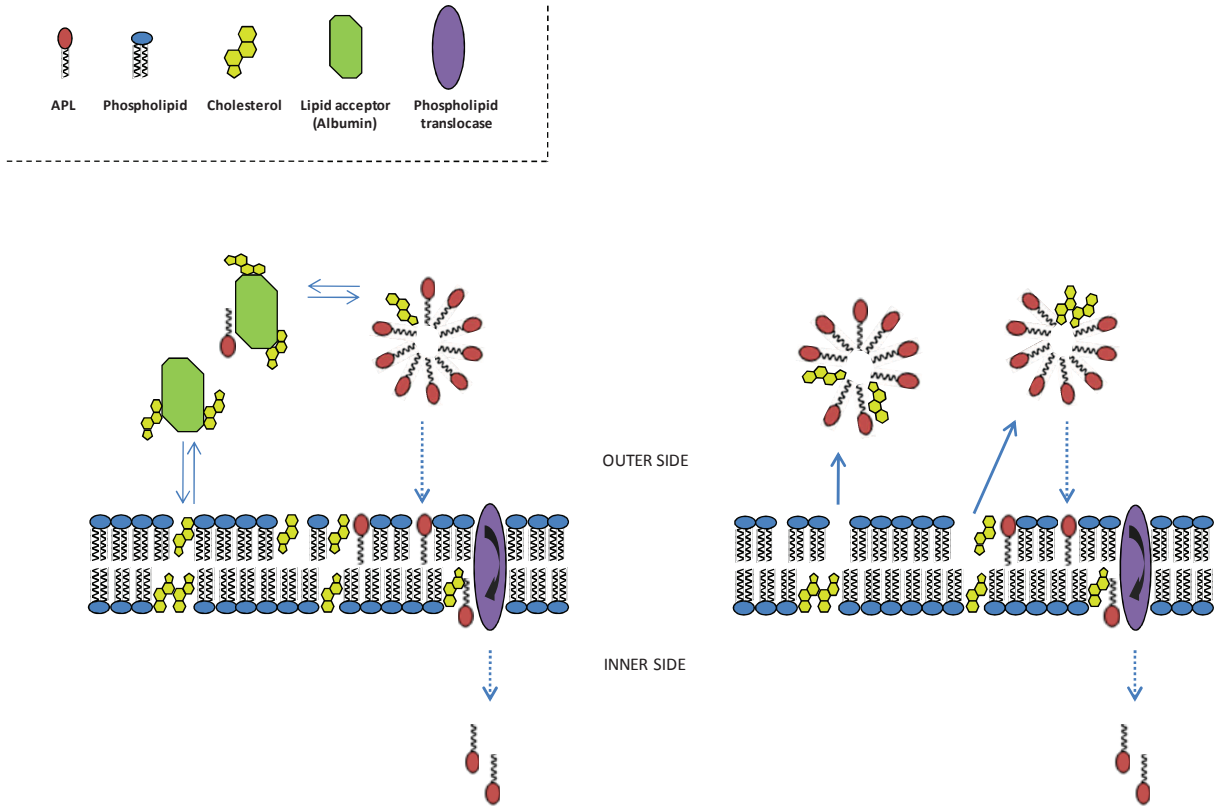

Figure 3. Alkylphopholipid interaction with cholesterol and insertion into membrane. When APLs are incubated in serum-containing medium, cholesterol from plasma membrane is swapped between plasma membrane and extracellular acceptors of serum such as albumin (left). In serum-free medium APLs promote cholesterol extraction affecting quickly the plasma membrane integrity (right). In both cases APLs can be inserted into plasma membrane and internalized by phospholipid translocases.

It is interesting to remark that either in the presence or absence of cholesterol/serum in the medium, APLs decrease cholesterol ester formation (i.e. cholesterol trafficking from plasma membrane to ER). This indicates that, although cholesterol efflux can contribute to the APL´s biological effect, their ability to impair cholesterol trafficking is independent to the one that induce its efflux; this suggests that APLs are able to get internalized into the cells and act as antiproliferative agents either if they promote the release of cholesterol or not. The proposal is that APLs insert into membranes (Busto et al., 2008; Steck and Lange, 2010) and

interfere with cholesterol transport from the plasma membrane to the ER and in addition they can act as efficient acceptors of cholesterol when there are no other physiological acceptors like lipophilic molecules from serum (Fig. 3). In regards to APLs internalization into the cell this process is not completely understood; some authors suggest that phospholipid translocases are involved (Barrat et al., 2009), indeed researchers from laboratory of Francisco Gamarro have cloned in Leishmania *donovani* a putative miltefosine transporter which behaves as a translocase and highly increase HePC and edelfosine internalization into the parasite (Pérez-Victoria et al., 2003). This posibility however, is not yet clear in mammals.

We have performed several studies on regards with the efficiency and action mechanism by which APLs extract cholesterol and have compared them with MβCD – one the traditionally used agents for extracting cellular cholesterol. Firstly, we report that APLs promote cholesterol sorting in a clearly more effective way than MβCD. Indeed, cholesterol replenishment of HepG2 cells after treatment with MBCD or APLs in absence of serum showed that MβCD-treated cells recover their morphology and cholesterol levels whereas APL-treated cells do not. Secondly, there is the fact that not only cholesterol but other lipids as phospho- or sphingo-lipids could be effluxed from the cell when working with extracellular lipophilic acceptors. On regards with APLs, cholesterol efflux entailed release of other lipids such as phospholipids and sphingolipids, although in a significantly smaller proportion than cholesterol. MβCD however, has not been shown to promote such effect on other lipids but cholesterol (Kilsdonk et al., 1995; Mahammad and Parmryd, 2008). Finally, cyclodextrins are reported to act as cholesterol acceptors or donors depending on the amount of cholesterol they are interacting with. For instance, in Fu5AH rat hepatoma cell cultures, complexes of cyclodextrin/cholesterol with molar ratios of 20/1 for MβCD or 70/1 for 2-Hydroxypropyl-β-cyclodextrin have shown to act as cholesterol donors (Christian et al., 1997). Although APLs are structurally different to cyclodextrines we went on to analyze if they could act as donor too. Results from chapter 4.6. show how HePC/cholesterol at 1/20 or edelfosine/cholesterol at 1/40 molar ratios are able to introduce cholesterol inside the HepG2 cells, instead of depleting it from them. It is noteworthy than, although these vesicles need to be very saturated in cholesterol, this is the first time that APLs are reported to be able to act as cholesterol donors, supporting the idea of that APL are organized in extracellular vesicles and can act as a acceptor/donor bidirectional system with the highest affinity for cholesterol.

Summarizing, all the results presented in this thesis support the idea that the mechanism by which APLs exert their antiproliferative action is highly linked with a drastic alteration of the cholesterol homeostasis inside cancer cells. Moreover, cell death processes such as cell cycle arrest or autophagy have been shown in APL-treated cancer cells which are related with signaling survival routes as PI3K/AKT, reported as well to be modulated by cholesterol cellular content. Although it has not been completely elucidated, it is quite clear that the alteration in cholesterol trafficking disrupts the cholesterol feedback mechanisms and so levels and location of cholesterol inside the cells are mismatched after APL-treatment. All this entails the death of cancer cells that are dependent on these pathways and therefore the modulation of cholesterol metabolism could certainly be a key that will help beating the cancer with lipid analogs that are currently used in clinics.

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Conclusions

6. Conclusions

1. The alkylphospholipids miltefosine, edelfosine, erucylphosphocholine and perifosine exert antiproliferative effects on HepG2 and U-87 MG cell lines in a time- and dose-dependent manner. The inhibition in cell growth is related with cell cycle arrest but not with unspecific cytolysis.

2. The analysis of tumor cells treated with alkylphospholipids by transmission electron microscopy has shown that autophagy is induced. Although it remains unclear, this process could be a protective mechanism from cells to overcome the cytostatic effects of alkylphospholipids.

3. It is clearly demonstrated that all alkylphospholipids share a common action mechanism on intracellular cholesterol trafficking that consist in inhibiting its arrival to the endoplasmic reticulum. This blockage is thought to affect a route that is non vesicular and independent of endocytosis since lysosomal markers do not colocalize with cholesterol in the performed fluorescence microscopy studies.

4. The inhibition of cholesterol transport to the endoplasmic reticulum caused by alkylphospholipids entails activation of transcription factor SREBP-2 and downstream genes such as HMGCR and LDLR. As a result, cholesterol biosynthesis is enhanced and the cholesterol, and some intermediates, are accumulated inside the treated cells. Some other processes such as cholesterol catabolism and efflux are repressed in HepG2 cells, contributing to increase of cholesterol inside the treated cells.

5. Alkylphospholipids alter the cholesterol/sphingomyelin ratio in plasma membrane and disturb lipid raft composition. In addition, the raft-dependent PI3K/AKT survival pathway is inhibited by these drugs; more specifically, PIP3 levels as well as AKT phosphorylation are affected by them. The modulation of AKT phosphorylation has been shown to be linked with cholesterol removal and replenishment from the plasma membrane.

6. Alkylphospholipids are able to interact with cholesterol and extract it from plasma membrane in extracellular serum-free medium. This effect is abolished in presence of lipid acceptors or cholesterol itself in the medium. However, inhibition of cholesterol trafficking is present in cells treated with alkylphospholipids in either free-serum or containing-serum media.

All the results in the presented thesis strongly support the conclusion that alkylphospholipids interfere with the arrival of cholesterol to endoplasmic reticulum, surpassing the strict mechanisms that cells have developed to regulate intracellular cholesterol levels. Since cholesterol is depleted in the endoplasmic reticulum, the site of cholesterol sensing, an homeostatic response is triggered in an attempt to increase the endogenous levels of cholesterol in cells that in fact have an excess of sterols.

6. Conclusiones

1. Los alquilfosfolípidos miltefosina, edelfosina, erucilfosfocolina y perifosina ejercen efectos antiproliferativos sobre las líneas celulares HepG2 y U-87 MG de manera proporcional al tiempo y dosis. Esta inhibición en el crecimiento celular está relacionada con la detención del ciclo celular, pero no con citolisis inespecífica.

2. El análisis de las células tumorales tratadas con alquilfosfolípidos mediante microscopía electrónica de transmisión ha demostrado que en la autofagia está inducida en dichas células. Aunque no está del todo claro, este proceso podría ser un mecanismo protector de las células para superar los efectos citostáticos de los alquilfosfolípidos.

3. Los resultados obtenidos claramente demuestran que todos los alquilfosfolípidos comparten un mecanismo de acción común sobre el tráfico intracelular de colesterol, que consiste en la inhibición de su llegada al retículo endoplasmático. Creemos que este bloqueo afecta a una ruta no vesicular e independiente de endocitosis puesto que los marcadores lisosomales no colocalizan con el colesterol en los de microscopía de fluorescencia realizados.

4. La inhibición del transporte de colesterol al retículo causada por alquilfosfolípidos implica la activación del factor de transcripción SREBP-2 y de genes diana tales como HMGCR y LDLR. Como resultado, la biosíntesis de colesterol aumenta y el colesterol y sus intermediarios se acumulan en las células tratadas. Algunos otros procesos, tales como el catabolismo de colesterol y su salida están reprimidos en las células HepG2, lo que contribuye al aumento de colesterol en las células tratadas.

5. Los alquilfosfolípidos alteraran la proporción de colesterol/esfingomielina en la membrana plasmática y perturban la composición de rafts lipídicos. Además, la vía de supervivencia PI3K/AKT, dependiente de raft, es inhibida por estos fármacos; de hecho los niveles de PIP3 y de fosforilación de AKT se ven afectados por ellos. Esta modulación de la fosforilación de AKT se ha demostrado estar relacionada con la eliminación o reposición del colesterol de membrana plasmática.

6. Los alquilfosfolípidos son capaces de interactuar con el colesterol y extraerlo de membrana plasmática en un medio extracelular libre de suero. Este efecto es suprimido en presencia de aceptores de lípidos o colesterol en el medio. Sin embargo, la inhibición del tráfico de colesterol sigue presente en las células tratadas con alquilfosfolípidos, ya sea en medio libre de suero o que lo contenga.

Todos los resultados presentados en esta tesis apoyan firmemente la conclusión de que los alquilfosfolípidos interfieren con la llegada de colesterol a retículo endoplasmático, sobrepasando los estrictos mecanismos que las células han desarrollado para regular los niveles de colesterol intracelular. Puesto que el colesterol se reduce en el retículo endoplasmático, el sitio de detección de colesterol, se activa una respuesta en un intento de aumentar los niveles endógenos de colesterol en las células que, en realidad, tienen un exceso de esteroles.