Cholesterol homeostasis and autophagic flux in perifosine-treated human hepatoblastoma HepG2 and glioblastoma U-87 MG cell lines

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1 Introduction

Brain and liver tumours are two of the most malignant types of cancers, posing major health problems and presenting especially difficult challenges to therapy. Evidence increasingly points to a connection between lipid metabolism and cancer, characterized mainly by an alteration in the mechanisms that regulate cholesterol homeostasis [1]. In this context, some researchers have proposed a potential strategy to treat glioblastoma by blocking the uptake of cholesterol into brain-cancer cells [2]. Other authors have reported that the active protrusion formed by invasive tumour cells is regulated by the cholesterol balance at the plasma membrane [3], and Kuzu et al. [4] have recently shown that some compounds mediate cancer-cell death by inhibiting intracellular cholesterol transport, leading to a homeostatic imbalance in the lysosomal-endosomal cell compartments.

Antitumour alkylphospholipids (APLs), such as perifosine, hexadecylphosphocholine (miltefosine), erucylphosphocholine, and edelfosine, are lipid analogues which exert antitumour activity against a broad spectrum of established tumour-cell lines [5]. There is growing interest in the biological activity of these lipid analogues as they do not interact with DNA but selectively inhibit the growth of transformed cells at concentrations that do not affect normal cells [6], and thus could well complement existing DNA-directed anticancer chemotherapies.

In previous studies, we have investigated the effects that these APLs exert upon cholesterol metabolism and have found that all of
them share a common mechanism of action to impede cholesterol from reaching the endoplasmic reticulum (ER), thus markedly reducing intracellular cholesterol esterification in HepG2 (human hepatoblastoma) and U-87 MG (human glioblastoma) cells. We have also demonstrated that APLs not only block cholesterol esterification in both cell lines but also increase its synthesis and internalization, with 3-hydroxy-3-methylglutaryl-CoA reductase and low-density lipoprotein receptor (LDLR) being up-regulated [7–9]. Consequently, exposure of these cells to APLs leads to a deregulation of cholesterol homeostasis. In relation to this, we have recently reported that exposure of U-87 MG cells to APLs substantially alters the intracellular lipid metabolism and induces autophagosome accumulation [9]. Autophagy is a process of bulk degradation that in mammals is important for the turnover of long-lived proteins and acts as a pro-survival mechanism during starvation. However, undue activation of autophagy can lead to a type of cell death that is distinct from apoptosis [10]. It is now recognized that the level of autophagy needs to be finely regulated within a certain range and that excessive or deficient autophagy may trigger various diseases. The interplay between autophagy and lipid metabolism is complex; autophagy regulates lipid metabolism, and alterations in intracellular lipid content are likely to be important in the autophagy pathway [11,12], but the underlying mechanisms remain unclear.

These observations prompted us to explore how perifosine, as a representative APL, could affect the autophagic process in human cancer-cell lines. Here, we report that perifosine impairs the autophagy flux related to a defect in cholesterol transport. Our study emphasizes that autophagy is a survival mechanism for HepG2 and U-87 MG cells, this being consistent with the fact that, by inhibiting the basal autophagic flux, perifosine interferes with the proliferation of these tumour cells.

2. Materials and methods

2.1. Materials

Foetal bovine serum (FBS) was obtained from the Cell Culture Company (Pascching, Austria). Minimal essential medium (MEM), thin-layer chromatography (TLC) plates, Fluoromount, chloroquine (CQ) and the protease-inhibitor cocktail were from Sigma-Aldrich (Madrid, Spain) and [1,2-3H(N)]cholesterol was from America Radiolabeled Chemicals, Inc (Saint Louis, MO, USA). Perifosine was obtained from Selleck Chemicals (Ontario, Canada). Cell Proliferation Reagent WST-1 kit was from Roche (Madrid, Spain). Polyclonal anti-human primary antibodies (B–actin, Beclin-1, CHOP/GADD153, GRP78/Bip, LC3) and Alexa Fluor 568 or horseradish peroxidase (HRP)-linked secondary IgGs were from Cell Signaling Technology (Danvers, MA, USA).

2.2. Cell culture

Human hepatoma HepG2 and human glioblastoma U-87 MG cell lines were obtained 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⁻¹ penicillin and 100 μg ml⁻¹ streptomycin, in a humid atmosphere with 5% CO₂ at 37 °C, and subcultured at a ratio of 1:10 once a week.

2.3. Assays for cell proliferation

Cells were seeded onto 96-well plates (10,000 cells/well) and maintained in MEM/10% FBS for 24 h. The culture medium was then replaced with fresh MEM/10% FBS and the cells incubated for 24 and 48 h without or with different concentrations of perifosine in the absence or presence of CQ. After incubation, 10 μl of Cell Proliferation Reagent WST-1 was added and the cells were incubated for 2 h at 37 °C and 5% CO₂. The absorbance of the formazan product in each well was measured directly in the plates at a wavelength of 450 nm using an ELx800 microplate reader (Biotek Instruments, Inc, Potton, UK). The reference wavelength was 600 nm.

2.4. Transmission electron microscopy

HepG2 and U-87 MG cells were seeded in 6-well plates and allowed to grow for 24 h. Subsequently, either 20 μM perifosine or a vehicle as control was added and left for 24 h in MEM/10% FBS. The cells were collected using trypsin and centrifuged at 1500 rpm for 5 min in MEM/10% FBS. The cell pellets were fixed in 2.5% glutaraldehyde plus 2% paraformaldehyde in 0.05 M cacodylate buffer for 4 h at 4 °C. The samples were washed three times with cacodylate buffer and postfixed in an aqueous solution of 1% OsO₄ containing 1% potassium ferrocyanide for 1 h at 4 °C in darkness. The following washes were carried out: 0.15% tannic acid in cacodylate buffer, cacodylate buffer, and water, all at room temperature. The samples were left in 2% uranyl acetate for 2 h and washed several times in water before being dehydrated at 4 °C in ethanol solutions rising from 50% to 100%. Next, the samples were placed into resin [EMbed 812/100% ethanol (1/1)] for 60 min at room temperature, 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 60 °C. Ultrafine sections (50–70 nm) were cut using a Leica Ultramicrotome R (Leica Microsystems, Barcelona, Spain) and contrasted using 1% aqueous uranyl acetate for 5 min and lead citrate in a CO₂-depleted atmosphere for 4 min [13]. A Libra 120 Plus electron microscope (Carl Zeiss microscopy, Jena, Germany) was used to study the sections.

2.5. Transport of cholesterol from the plasma membrane to the endoplasmic reticulum

The cells were seeded in 12-well plates at 60–70% confluency and cultured in MEM/10% FBS in the absence or presence of 20 μM perifosine and/or 20 μM CQ for 6 and 24 h. After treatment, the medium was removed and replaced with fresh medium containing 1 μg of [1,2-3H(N)]cholesterol for 60 min at room temperature to label the plasma membrane [14]. The cells were washed twice with PBS containing 0.5 mg/ml of BSA prewarmed to 37 °C to remove any unincorporated label. The cells were then incubated at 37 °C in MEM for 60 min, and afterwards the medium was removed and the cellular lipids were extracted. Cholesterol and cholesteryl esters were separated by TLC using a solvent of n-hexane/ethyl ether/acetic acid (80:20:2, v/v/v). The spots were rendered visible by exposure to iodine vapour and radiometric measurements of scraped lipid spots were made by liquid scintillation using a Beckman 6000-TA counter (Madrid, Spain).

2.6. Immunoblotting analysis

Cells growing in log-phase were incubated for 6 and 24 h with MEM/10% FBS in the absence (PBS as vehicle) or presence of the assayed compounds. The cells were washed twice, scraped into ice-cold PBS (pH 7.4) and centrifuged at 100 × g for 10 min at 4 °C. The 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, 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 used; an aliquot was taken to determine protein content...
concentration. Equal quantities of lysate protein were separated by SDS-PAGE and transferred onto polyvinylidene difluoride membranes. Pre-stained protein molecular weight 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 (1:1000) in 3% BSA-blocking buffer at 4°C overnight. After several washes in TBS containing 0.05% Tween-20, the membranes were incubated with the corresponding HRP-conjugated IgG (1:2000) as a 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, the blots were probed with polyclonal anti-human β-actin Ig (1:1000) 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).

2.7. Immunofluorescence analysis of LC3

HepG2 and U-87 MG cells were seeded on sterile microscopy slides at 5000 cells/chamber and allowed to adhere and grow for 24 h. The cells were treated with vehicle, 20 μM perifosine, 20 μM CQ or both agents in MEM/10% FBS. After 24 h they were fixed in 4% p-formaldehyde for 30 min at room temperature, washed 3 times with PBS, and permeabilized with 0.3% Triton X-100. Blocking was performed in PBS/10% FBS for 1 h at room temperature, after which the cells were incubated overnight at 4°C in PBS/10% FBS containing anti-LC3 primary antibody at 1:100. After washing, Alexa Fluor 568-conjugated secondary antibody was added for 60 min at 1:500 and finally washed once more. The slides were mounted using Fluoromount and visualized under a high-speed confocal microscope (A1 model, Nikon, Barcelona, Spain). Pictures were processed using ImageJ software.

2.8. Statistics

The results are expressed as means ± SEM. A one-way ANOVA was conducted with post hoc comparisons by Scheffe’s test (SPSS 13.0). 

2.9. Other analyses

Cell-protein content was determined by Bradford’s method [15] using BSA as standard.

3. Results

3.1. Ultrastructural alterations produced by perifosine

We analyzed the ultrastructure of HepG2 and U-87 MG cells in control and perifosine-treated cultures, using transmission electron microscopy (TEM) to study the morphologic alterations induced by perifosine. Untreated HepG2 cells (Fig. 1, inset A) showed a homogeneous cytoplasm with many mitochondria of dense matrix, well-developed rough ER, and an abundance of lipid bodies. Numerous surface microvilli and endocytic vesicles were also visible. Treatment with 20 μM perifosine for 24 h produced an intense cytoplasmic vacuolization corresponding to a notable dilatation of the ER cisterns, suggesting the presence of ER stress (Fig. 1, insets B, C). Also visible were double-membrane vesicles that matched autophagic bodies engulfing cytoplasmic material and cell organelles (Fig. 1, inset D).

Control U-87 MG cells were clearly morphologically different from HepG2 cells, with a more rounded shape and lower surface microvilli (Fig. 2, inset A). This cell type presented small...
mitochondria with a dense matrix and scant rough ER (Fig. 2, inset B). Remarkably, after 24-h exposure of U-87 MG cells to 20 μM perifosine the mitochondria appeared to be affected, showing clearly dilated cristae (Fig. 2, inset C) whilst the cytoplasm was highly vacuolized, due mainly to a significant increase in double-membrane bound structures (Fig. 2, insets B, D). These vacuoles contained cell debris and recognizable cytoplasmic organelles, suggesting they were autophagic in nature.

3.2. Analysis of the endoplasmatic reticulum stress response in HepG2 and U-87 MG cells exposed to alklyphospholipids

After TEM analysis, we looked for evidence of ER stress induced by APLs in HepG2 and U-87 MG cells. Experiments were carried out to evaluate the effect of 6- and 24-h treatment with perifosine on the protein levels of the apoptotic transcription factor CHOP (C/EBP homologous protein)/GADD153 (growth arrest and DNA damage-inducible gene 153), an acute ER stress marker [16], and the ER chaperone GRP78/BiP, a low-grade chronic ER stress marker which regulates the adaptive unfolded protein response (UPR) mechanism [17]. Cells treated with tunicamycin (TN) were included as a positive control, and other APLs such as miltefosine and edelfosine were also assayed. As shown in Fig. 3A, the incubation of hepatoblastoma HepG2 cells with diverse APLs for 6 h led to a 2–3 fold increase in CHOP expression as compared to the untreated cells (where it was expressed at a basal level); high levels of CHOP were maintained after 24-h exposure to perifosine or edelfosine. Treatment with TN, a powerful ER stress inducer, caused similar increases in CHOP expression at both exposure times. Levels of GRP78 were not affected by APLs in these cells; under the same conditions, incubation with TN induced a robust expression of GRP78.

Exposure of glioblastoma U-87 MG cells to APLs for up to 24 h did not induce CHOP expression with respect to basal levels. GRP78 protein expression did not change after incubation of these cells with APLs, in a similar way to the lack of effect in HepG2 cells. Treatment with TN, however, caused a higher expression of both ER-stress protein markers, CHOP and GRP78, in the U-87 MG cells (Fig. 3B).

Taken together, these results suggest that perifosine and other APLs cause an early activation of ER stress in HepG2 cells, whereas no feedback mechanism through UPR activation occurs to alleviate this stress and restore ER homeostasis. However, APLs failed to provoke ER stress in the U-87 MG cell line.

3.3. Alteration in autophagic flux induced by perifosine

To confirm changes in autophagy after treatment of cells with perifosine, we quantified levels of the key autophagy-pathway protein LC3 using Western blotting. LC3 is a small cytosolic ubiquitin-like molecule that is recruited by autophagosomes when autophagy is induced. Here, it is conjugated to a lipid, phosphatidylethanolamine, which can be readily visualized by increased electrophoretic mobility. Therefore, the conversion of LC3 (form I) to its lipidated form (LC3-II) augments during autophagy. However, an increase in autophagosomes and LC3 lipidation, though consistent with autophagy stimulation, could also result from a block in the late stages of autophagy, for example, the fusion of autophagosomes with lysosomes, i.e. autophagic flux inhibition. To discriminate between these two possibilities, we determined LC3-II levels in both control and perifosine-treated cells in the absence or presence of the lysosomotropic basifying agent CQ, which blocks lysosomal degradation.

Fig. 4 shows that, from the relative density of the LC3-II bands normalized to β-actin, the treatment of HepG2 cells with perifosine for 6 and 24 h resulted in higher levels of LC3-II (by 39% and 76%, respectively), compared to the controls. In addition, as expected, CQ treatment raised the levels of the LC3-II autophagic
marker; on the other hand, following lysosomal inhibition by CQ, LC3-II accumulation was increased no further by co-treatment with perifosine. With regard to U-87 MG cells, the same figure reflects that, although after 6 h of perifosine exposure the LC3-II levels had increased by up to 70% above those of the untreated cells, when treatment was prolonged over a 24-h period the levels of this autophagic marker remained unchanged compared to the controls. Again, the incubation of cells with CQ clearly raised the levels of the lipidated form of LC3. Notably, the LC3-II content in U-87 MG cells exposed for 6 h to both CQ and perifosine was higher...
than that of the cells treated with either agent alone, whereas after 24 h no significant differences were detected in the levels of this marker between cells exposed to CQ alone and those treated with CQ plus perifosine.

An easy, effective method to infer the real autophagic flux has been proposed, in which LC3-II levels are subtracted in the presence/absence of lysosomal inhibitors. Accordingly, the difference in the levels of LC3-II in the presence or absence of these inhibitors will reflect the net amount of this protein delivered to the lysosomes. If a compound raises LC3-II levels in the absence of inhibitor, but there is no further rise in its presence, then it can be inferred that there is a blockage in LC3-II degradation [18–20]. Fig. 5 illustrates the results of this analysis, showing that treatment with perifosine for 6 and 24 h decreases LC3-II degradation and thus the autophagic flux in HepG2 cells (34% and 21%, respectively). In a different manner, the exposure of U-87 MG cells to perifosine for 6 h increases the autophagic flux (71%), but when the treatment is continued for 24 h this flux is clearly inhibited (23%).

These data were further confirmed by immunofluorescence microscopy. As shown in Fig. 6, untreated HepG2 and U-87 MG cells present a faint staining of LC3 with diffuse distribution within the cytoplasm, consistent with the cytoplasmic delipidated form of the protein. On the other hand, perifosine treatment for 24 h produces a more punctuated staining pattern, suggesting a vesicular distribution of active LC3 isoform. Additionally, CQ-treated cells present a more evident vesicular LC3 distribution in the cytoplasm. No significant differences were detected between the cells exposed to CQ with or without perifosine. These effects in LC3 distribution suggest that treatment with perifosine and/or CQ promotes an accumulation of autophagic middle-stage LC3-enriched vesicles, i.e. autophagosomes.

Also, we performed Western blotting to determine Beclin-1, the key protein for autophagosome assembly in the Bcl-2/Beclin-1-dependent autophagy pathway. The results (Fig. 4) show that, compared to the control, the levels of this protein were not significantly changed by 6 or 24 h perifosine and CQ treatments in HepG2 or in U-87 MG cells, and therefore we discard the hypothesis that this APL induces autophagy.

3.4 Effects of perifosine and chloroquine on cell growth

Next, we examined whether cell proliferation is affected by the accumulation of autophagosomes associated with impaired autophagic degradation induced by perifosine. To do so, we treated cells with perifosine alone or in combination with CQ. As expected, perifosine clearly diminished cell proliferation in HepG2 cells, and the co-treatment with CQ and perifosine markedly reduced cell growth compared to perifosine alone, after 24 h and 48 h (Fig. 7A). In U-87 MG cells, perifosine treatment again significantly diminished cell numbers, but CQ had only strengthened the antitumoural activity of perifosine slightly after 48 h exposure (Fig. 7B). From these results, we conclude that inhibition of the autophagy pathway in both cell lines sensitizes cells to perifosine.

Fig. 4. Effects of perifosine and chloroquine on LC3-II and Beclin-1 protein levels in HepG2 and U-87 MG cells. Cells were incubated with MEM/10% FBS without any additions (control) or containing 20 μM perifosine and/or 20 μM chloroquine for 6 or 24 h. Cell lysate samples were collected and analyzed by immunoblotting to determine the content of LC3-II and Beclin-1, as described in “Methods” section. Western blot analyses of LC3 isoforms, Beclin-1 and β-actin (used as loading control) in HepG2 (A) and U-87 MG (B) cells are shown. The bands were scanned and arbitrary units were assigned by densitometric analysis. LC3-II protein levels in the samples were normalized to their respective β-actin levels. The figure shows a representative experiment repeated three times. *p < 0.05 as compared with the respective control.
3.5. Effects of perifosine and chloroquine on the traffic of cholesterol from the plasma membrane to the endoplasmic reticulum

Previous work in our laboratory has shown that both HepG2 and U-87 MG cells, when exposed to APLs for 24 h, exhibit impaired cholesterol transport from the plasma membrane to the ER, prompting a robust increase in their cholesterogenic capability and leading to a significant accumulation of intracellular cholesterol [7,8,14].

We have now extended these studies to analyze the effects of 6 and 24 h perifosine and/or CQ exposure on the intracellular cholesterol traffic in these cells. Knowing that an appropriate experimental procedure to study any interference in this pathway is to analyze the rate of cholesterol ester synthesis from plasma-membrane cholesterol [9,21], we incubated the cells with perifosine, CQ or the combination of the two, and then labelled the plasma membrane with radioactive cholesterol for 60 min and determined the radioactivity appearing in the esterified cholesterol.

Fig. 5A illustrates how the perifosine and/or CQ treatments led to a decrease in cholesteryl ester formation in HepG2 cells after both 6 h and 24 h, thus demonstrating an inhibition of cholesterol transport from the plasma membrane to the ER. It bears noting that, although exposure of U-87 MG cells to perifosine and/or CQ for 24 h produced a similar inhibition in the arrival of cholesterol at the ER, when these treatments lasted only 6 h the traffic of cholesterol from the plasma membrane to the ER appeared to be undisturbed (Fig. 5B). These results suggest a relationship between the deregulation of cholesterol homeostasis and the inhibition of the autophagic flux produced by perifosine.

4. Discussion

In this study, we investigate the effects of perifosine on autophagy and its crosstalk with cholesterol homeostasis, ER stress, and cell proliferation in HepG2 and U-87 MG cells. Our main goal is to compile additional information on the mechanisms by
which perifosine takes effect and thereby to open new strategies to improve its efficiency in antitumor therapy.

Our results show that the exposure of cells to perifosine results in an accumulation of autophagosomes that is not due to increased autophagosome synthesis but rather to a blockage in the autophagic flux. We found no alteration in the upstream events regulating autophagosomal synthesis, such as the levels of Beclin-1 after perifosine exposure. Accordingly, this APL does not stimulate the autophagy process, suggesting that perifosine may be inhibitory in the late stages of autophagy. Our data contrast with previous findings attributing the increase in LC3-II after perifosine treatment to an induction of autophagy [22,23]. However, our findings agree with those of other studies in which altered cholesterol homeostasis has been correlated with autophagy [4]. In fact, a complex interplay between autophagy and lipid metabolism has been demonstrated [11,12,24]. Notably, our laboratory previously reported (for the first time) that exposure of tumour cells for 24 h to APLs interferes with the intracellular transport of cholesterol from the plasma membrane to the ER. Consequently, cholesterol is depleted in the ER, the site where the levels of this sterol are sensed and the mechanisms to increase cholesterol levels are triggered, thus causing a constant activation of the sterol regulatory element-binding protein 2 (SREBP-2) transcription factor, which is translocated to the nucleus, activating the transcription of cholesterogenic genes and LDLR. Therefore, cells exposed to APLs accumulate intracellular cholesterol [7–9]. As we demonstrate in the present study, not only perifosine but also CQ affects the intracellular traffic of cholesterol. No available reports have shown that CQ, a lysosomotropic agent that affects lysosomal activity and induces autophagosome accumulation, also alters the transport of cholesterol from plasma membrane to the ER. In our opinion, the above-described results strongly suggest a direct relationship between the impairment in cholesterol traffic from the plasma membrane to the ER and the inhibition in autophagic flux. Thus, in both U-87 MG and HepG2 cells when cholesterol traffic is impeded, autophagic flux becomes inhibited. In this sense, it should be noted that in U-87 MG cells exposed to perifosine for just 6 h the transport of cholesterol from the plasma membrane to the ER is unaltered and autophagic flux is not inhibited.

Corroborating our results, other researchers have reported that alterations in intracellular cholesterol traffic, as occurs after U18666A treatment and Niemann Pick C1 (NPC1) disease, boost the levels of LC3-II together with an accumulation of cholesterol [25]. Similarly, Maetzel et al. [26] demonstrated that in both NPC1-deficient hepatic and neural cells that accumulate cholesterol
the late endosomal-lysosomal compartment, autophagosome maturation is impaired, increasing the autophagosome content. The process of autophagosome/lysosome fusion severely depends on the intracellular cholesterol content, although the way in which cholesterol participates in the autophagosome maturation remains unknown. Whereas a moderate cholesterol increment has been shown to stimulate autophagic flux, the fusogenic ability of the autophagic-lysosomal compartments is attenuated under conditions of chronic lipid exposure [27]. Thus, variable effects on autophagy have been reported for cholesterol [28]. An acute cholesterol stimulus such as acetylated human low-density lipoprotein (AcLDL) loading in macrophages [29] or free cholesterol treatment of smooth-muscle cells [30] can increase autophagy, while a reduced level of cholesterol in the membrane of either autophagosomes or lysosomes results in a dramatic reduction in fusion. These findings support the hypothesis that cholesterol from both vesicles participates in membrane fusion [27,31].

It is widely recognized that perifosine inhibits Akt enzyme, thereby disrupting the phosphatidylinositol 3-kinase/Akt pathway/mTOR signalling [32,33], this signalling pathway being involved in its antiproliferative action. However, in the last decade, several reports have suggested a role for autophagy in cell survival at different stages of tumour development and in the tumour-cell response to anticancer therapy [34]. In addition to its conventional role in cell survival, autophagy can also be a death promoter, in particular when autophagy is extensive. Therefore, it is reasonable to assume that the inhibition of the autophagy process caused by perifosine might be a determining factor in the antiproliferative action of this APL. On analysing the effects of perifosine on cell proliferation, we found that this APL displayed growth-inhibitory activity in both HepG2 and U-87 MG cell lines, which became more pronounced as treatment time lengthened. With respect to U-87 MG cells, few reports describe the effects of APLs on the proliferation of glioblastoma cell lines. Our results agree with those of Qin et al. [23], who showed that perifosine dose-dependently reduced cell proliferation in U-87 MG cells. Therefore, in our study perifosine was found to impair autophagic flux in both cell lines whereas it caused only a ER stress response in HepG2 cells, indicating that this effect does not seem to play a crucial role in the inhibition of cell proliferation.

Under our experimental conditions, treatment with perifosine combined with CQ further decreased cell proliferation compared to perifosine alone. Our study emphasizes that autophagy is required for the survival of HepG2 and U-87 MG tumour cells, and is consistent with the fact that inhibiting the basal autophagy flux interferes with the maintenance of these cells.
In conclusion, we report perifosine as a novel inhibitor of autophagy flux. Perifosine inhibits the autophagic process apparently in relation to its capacity to impede intracellular cholesterol transport. Our study might be relevant for anticancer therapy because tumour cells activate autophagy to survive, whilst the obstruction of the autophagic flux inhibits cell proliferation. Further research into the precise mechanisms of autophagic maturation and the role of cholesterol may provide new insights to help us understand the signalling machinery underlying the antiproliferative action of perifosine and other APLs.

Authors’ contribution

P.R.-M., J.M.J.-L., M.P.C. and C.M. designed, conceived, performed and analyzed the experiments. A.R. carried out TEM experiments. M.P.C. and C.M. wrote the manuscript.

Conflicts of interest

The authors declare that there are no conflicts of interest associated with this manuscript.

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