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Lipid rafts and metabolic energy differentially determine uptake of anti-cancer alkylphospholipids in lymphoma versus carcinoma cells

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ABSTRACT

Perifosine is a member of the class of synthetic alkylphospholipids (APLs) and is being evaluated as anti-cancer agent in several clinical trials. These single-chain APLs accumulate in cellular membranes and disturb lipid-dependent signal transduction, ultimately causing apoptosis in a variety of tumor cells. The APL prototype edelfosine was previously found to be endocytosed by S49 mouse lymphoma cells via lipid rafts. An edelfosine-resistant cell variant, $S49^{AR}$, was found to be cross-resistant to other APLs, including perifosine. This resistance was due to defective synthesis of the raft constituent sphingomyelin, which abrogated APL cellular uptake. Sensitivity of S49 cells to edelfosine was higher than perifosine, which correlated with a relatively higher uptake. Human KB epidermal carcinoma cells were much more sensitive to APLs than S49 cells. Their much higher APL uptake was highly dependent on intracellular ATP and ambient temperature, and was blocked by chlorpromazine, independent of canonical endocytic pathways. We found no prominent role of lipid rafts for APL uptake in these KB cells; contrary to $S49^{AR}$ cells, perifosine-resistant KBr cells display normal sphingomyelin synthesis, whereas APL uptake by the responsive KB cells was insensitive to treatment with methyl-β-cyclodextrin, a cholesterol-sequestrator and inhibitor of raft-mediated endocytosis. In conclusion, different mechanisms determine APL uptake and consequent apoptotic toxicity in lymphoma versus carcinoma cells. In the latter cells, APL uptake is mainly determined by a raft- and endocytosis-independent process, but metabolic energy-dependent process, possibly by a lipid transporter.

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Abbreviations: APL, alkylphospholipid; MβCD, methyl-β-cyclodextrin; PC, phosphatidylcholine; ErPC, erucylphosphocholine; SM, sphingomyelin; PBS, phosphate-buffered saline; TLC, thin-layer chromatography.

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1. Introduction

The group of synthetic single-chain alkylphospholipids (APLs) is a heterogeneous class of anti-cancer agents that exert cytotoxic effects against a wide variety of tumors [1–5]. In contrast to classical chemotherapeutic drugs, APLs do not target DNA but primarily act at the level of cell membranes. This distinct mode of action, which includes inhibition of phospholipid turnover and interference with lipid-dependent signaling pathways, make APLs attractive candidates for combination therapy with classical anti-cancer agents and ionizing radiation [6,7]. Several APLs are currently under investigation as radiosensitizers, since they have been shown to enhance radiation-induced cell death, both *in vitro* [6,8–13] and *in vivo* [11].

The prototype of these APL compounds is 1-*O*-octadecyl-2-*O*-methyl-*rac*-glycero-3-phosphocholine (Et-18-OCH_3; edelfosine), an ether-linked phospholipid with a glycerol backbone. Other members of the APL family lack this glycerol backbone, their alkyl chain being directly esterified to a phosphocholine moiety or analogue thereof, for example hexadecylphosphocholine (miltefosine), erucylphosphocholine (ErPC, which is intravenously applicable) and octadecyl-(1,1-dimethyl-4-piperidinio-4-yl)-phosphate (compound D-21266; perifosine [14]). This latter compound has been tested in phase I trials in patients with solid tumors as single agent [15,16] and in combination with radiotherapy [17] and is currently being evaluated in phase II studies.

In general, tumor cells can gain resistance to cytotoxic agents via multiple mechanisms, such as increased drug metabolism, enhanced drug efflux and reduced drug accumulation. There is no evidence for increased APL degradation. Similar to edelfosine [18], we found that perifosine is almost completely metabolically stable, both *in vitro* and *in vivo* [19]. Reduced APL uptake, however, is often observed in tumor cells that have gained resistance to these synthetic lipids [18,20–22]. Conversely, the high sensitivity of KB squamous cell carcinoma to APLs is correlated with a high degree of drug accumulation [19]. The precise mechanism by which APLs are internalized has long been a matter of debate. Whereas several investigators have reported a reduced uptake by tumor cells after treatment with pharmacological inhibitors of endocytosis [23–25], others failed to show a correlation between resistance to APL and endocytosis [26,27].

Our group has recently demonstrated, in S49 lymphoma cells, that edelfosine and perifosine accumulate in sphingolipid- and cholesterol-enriched plasma membrane microdomains, known as lipid rafts [28,29]. The drugs are then rapidly internalized via these domains in S49 cells; whereas this process is disturbed in the edelfosine-resistant cell variant $S49^{AR}$ [22,29]. This abrogated uptake of edelfosine was linked to reduced levels of sphingomyelin (SM), an essential raft constituent. Recently, we showed this defect to occur at the level of SM synthase 1 expression [30].

Although lipid rafts are clearly involved in APL uptake by lymphoma cells, their role in the uptake by solid tumors remains unknown. The human squamous cell carcinoma KB has previously been studied as an APL-responsive tumor model, both *in vitro* [19,31] and *in vivo* [11,32]. Here, we compared the APL uptake and toxicity in the S49/$S49^{AR}$ lymphoma with the KB carcinoma model and an APL-resistant cell variant, KBr. APL resistance was induced differently in these cells. $S49^{AR}$ cells were selected after continuous edelfosine treatment [33] whereas KBr was generated after chemical mutagenesis and subsequent continuous culturing in the presence of perifosine. In both cell models, we find a correlation between cellular APL uptake and sensitivity, as well as cross-resistance to other APLs. However, contrary to S49 cells, APL uptake by KB cells is predominantly raft- and endocytosis-independent but more dependent on metabolic energy, possibly an ATP-dependent lipid transporter.

2. Materials and methods

2.1. Materials

[Methyl-^{14}C]choline chloride (56 mCi/mmol) and L-lyso-3-phosphatidylcholine, 1-[1-^{14}C]palmitoyl ([^{14}C]LysoPC, 56 mCi/mmol; code CFA633) were purchased from Amersham Pharmacia Biotech. Erucylphosphocholine (ErPC), compound D-21805 (with arsenic substituting the choline nitrogen atom), perifosine (octadecyl-(1,1-dimethyl-4-piperidinio-4-yl)-phosphate; compound D-21266) and [^{14}C]perifosine (30.9 mCi/mmol) were kindly provided by Zentaris GmbH (Frankfurt, Germany). Edelfosine (Et-18-OCH_3) was from Kamiya Biomedical Company (Seattle, WA). [^{3}H]Edelfosine (39 Ci/mmol) was synthesized by Moravek Biochemicals (Brea, CA). Miltefosine (hexadecylphosphocholine) was from Cayman Chemical (Ann Arbor, MI). Reagents for lipid extraction and subsequent analyses, as well as Silica 60 TLC plates (20 cm × 20 cm) were from Merck (Darmstadt, Germany). BODIPY-lactosylceramide, Alexa-488-labeled transferrin and FITC-dextran were from Molecular Probes (Leiden, The Netherlands). All other chemicals were from Sigma (Zwijndrecht, The Netherlands).

2.2. Cells cultures

The human epidermal carcinoma KB cells (ATCC number CCL-17) and their perifosine-resistant variant KBr cells were kindly provided by Dr. F. Gamarro (Granada, Spain). The KBr cells (also named KB PER^{R} clone 10) were obtained by mutagenesis with ethyl-methanesulfonate, followed by intervals of incubation with increasing concentrations of perifosine, as described in detail elsewhere (F. Munoz–Martinez, C. Torres, S. Castanys and F. Gamarro, in preparation). KB and KBr were maintained in Dulbecco's modified Eagle's medium (Gibco, Rockville) supplemented with 10% (v/v) fetal bovine serum (Life Technologies) and antibiotics. Mouse S49.1 lymphoma cells (S49) were grown in Dulbecco's modification of Eagle medium, containing high glucose and pyruvate, supplemented with 8% fetal calf serum, 2 mM L-glutamine and antibiotics. Edelfosine-resistant variants ($S49^{AR}$) were isolated in two selection rounds of growth in 15 μM edelfosine (Et-18-OCH_3) for 72 h, followed by plating in semi-solid medium and isolation of colonies of surviving cells, as described by Smets et al. [33]. The selective $S49^{AR}$ clone could be grown continuously in 15 μM edelfosine with a doubling time of 12 h, similar to the parental S49 cells. Experiments with $S49^{AR}$ cells were performed with cells grown without the selection

agent for at least 1 week. All cell lines were tested negative for mycoplasma.

2.3. Phospholipid synthesis

KB/KBr cells (2.5×10^5 per well in 6-well plates) and S49/S49^{AR} cells (1×10^6 per well in 6-well plates) were incubated overnight and labeled for 8 h with 1 μCi/mL [methyl-^{14}C]choline chloride to measure SM synthesis. For PC synthesis from exogenous LysoPC, cells were incubated for the indicated times with 0.025 μCi [^{14}C]LysoPC. Cells were harvested, washed and fixed in methanol. Lipids were extracted with chloroform/methanol (1:2, v/v) and phase separation was induced using 1 M NaCl. The organic phase was washed in a solution of methanol/H_2O/chloroform (235:245:15, v/v/v) and separated by silica TLC, using choroform:methanol:acetic acid:water, 60:30:8:5, v/v/v/v). Radioactive phospholipids, among which [^{14}C]PC and [^{14}C]SM, were identified using internal standards and quantified using a Fuji BAS 2000 TR Phosphor-Imager.

2.4. Apoptosis assay

KB/KBr cells were plated in 6-well plates (1×10^5 cells/well) and allowed to attach overnight, whereas S49/S49^{AR} cells were plated in 96-well plates (1×10^5 cells/well) before incubation for indicated time periods with APLs. Cells were washed in PBS and incubated at 4 °C in 0.1% (w/v) sodium citrate, 0.1% (v/v) Triton X-100 and 50 μg/mL propidium iodide [34]. Fluorescence intensity of propidium iodide-stained sub-nuclear DNA fragments was determined by FACScan analysis (Becton Dickinson, San Jose, CA). The data were fitted to a sigmoidal concentration–response curve and EC_{50} calculation was done using GraphPad Prism version 4.00 for Windows (GraphPad Software, San Diego, CA).

2.5. APL uptake and ATP depletion

Cells were plated in 12-well dishes (S49/S49^{AR}-1×10^6 cells/mL; KB/KBr, 5×10^4 cells/well) and incubated overnight. Cells were pretreated for 30 min with 2.5 mg/mL methyl-β-cyclodextrin or 20 μM chlorpromazine, where indicated. Hereafter, cells were incubated with [^{14}C]perifosine (0.03 μCi/mL, 1 μM) or [^{3}H]edelfosine (0.15 μCi/mL, 1 μM). At appropiate time points, cells were washed with PBS, subsequently lysed in 1 M NaOH and diluted in Ultima Gold scintillation liquid (Perkin–Elmer, Wellesley, MA) for scintillation counting in a TRI-CARB liquid scintillation analyzer. ATP depletion was obtained by pre-incubation for 30 min with DMEM lacking D-glucose and sodium pyruvate and containing 20 mM 2-deoxy-D-glucose and 10 mM sodium azide. Statistical analysis of the data was performed using the Student's *t*-test.

2.6. K^+ depletion and ^{125}I-EGF internalization

KB cells were incubated for 5 min in DMEM/H_2O 1:1 (v/v) at 37 °C, followed by a 60 min incubation at 37 °C in minimal medium without K^+ (20 mM Hepes at pH 7.5, 140 mM NaCl, 1 mM $CaCl_2$, 1 mM $MgSO_4$, 5.5 mM glucose) and 0.5% (w/v) BSA. Control cells were incubated in minimal medium supplemented with 10 mM KCl. Cells were incubated for 5 min with 1 ng/mL ^{125}I-EGF (epidermal growth factor) (684 mCi/mmol; from Amersham) or for 1 h with radiolabeled APL (see above). Membrane-bound EGF was stripped off by washing with acidified PBS (HCl, pH 2.1) for 2 min at 4 °C. Radioactivity in cells was measured by scintillation counting.

2.7. Isolation of lipid rafts

A lipid raft fraction was prepared by detergent extraction of cells and sucrose gradient centrifugation for the determination of incorporation of radiolabeled APL in lipid rafts [22]. S49 cells were grown to a density of 2.0×10^6/mL, incubated with [^{14}C]perifosine (0.03 μCi/mL; 20 μM) or [^{3}H]edelfosine (0.15 μCi/mL; 15 μM). KB cells were plated in 8 cm diameter dishes (5×10^6 per dish—2 dishes for each raft isolation), allowed to attach overnight and incubated with [^{14}C]perifosine or [^{3}H]edelfosine (0.02 μCi/mL; 1 μM) for 30 min. Cells were washed twice with ice-cold PBS, solubilized into 2 mL of ice-cold MBST buffer (25 mM MES, 150 mM NaCl, 1% Triton X-100) and homogenized with a tight fitting Dounce homogenizer (10 strokes). The extract was adjusted to 40% sucrose by the addition of 2 mL of 80% sucrose in MBS (lacking Triton X-100) and put on the bottom of an ultracentrifuge tube. A discontinuous sucrose gradient was prepared by overlaying 5 mL of 30% sucrose and 3 mL of 5% sucrose (both in MBS), respectively. The tubes were centrifuged at 39,000 rpm in a SW41 rotor for 20 h at 4 °C and 12× 1.0 mL fractions were collected manually from the top of the gradient.

2.8. Confocal microscopy

KB/KBr cells (1×10^5) were grown on glass coverslips overnight. Cells were incubated in serum-free DMEM for 30 min and subsequently incubated for 30 min with BODIPY-lactosylceramide (5 μM), Alexa-488-labeled transferrin (10 μg/mL) or FITC-dextran (2 μg/mL). The cells were subsequently washed twice with ice-cold PBS and fixed for 15 min in 4% formaldehyde/PBS. Thereafter, cells were washed with PBS and mounted in Vectashield (Vector Laboratories, Ltd., Peterborough, England). Microscopy was done using a TCS SP2 confocal microscope (Leica Microsystems B.V., Rijswijk, The Netherlands).

3. Results

3.1. Drug-resistance induced by either edelfosine or perifosine causes cross-resistance to other APLs

We used two tumor cell models, the mouse lymphoma S49 with its edelfosine-resistant variant S49^{AR} [33] and the human squamous cell carcinoma KB with its perifosine-resistant variant KBr to study APL uptake and consequent cellular sensitivity or resistance towards apoptosis induction by these drugs. The dose–response curves reveal that S49 cells are approximately fivefold more sensitive to edelfosine than to perifosine. The data furthermore indicate that the edelfosine-resistant S49^{AR} are cross-resistant to perifosine. However, the difference in sensitivity towards perifosine compared to

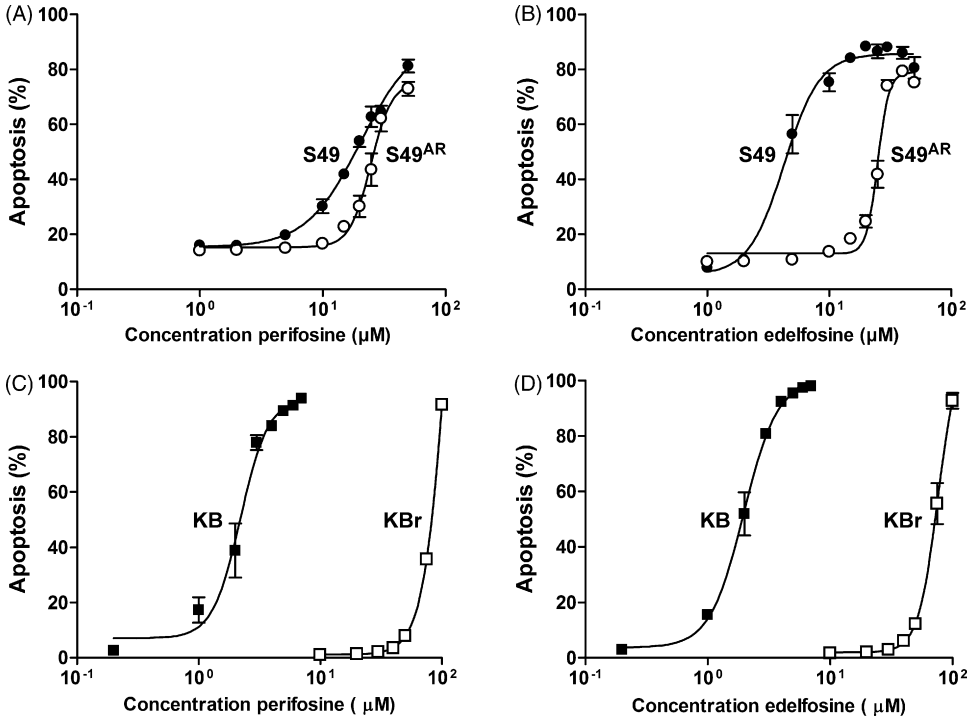

Fig. 1 – Differential sensitivity of S49 and KB cells and their APL-resistant cell variants S49^{AR} and KBr, respectively, to perifosine or edelfosine. Dose-dependent induction of apoptosis in S49 (solid circles) and S49^{AR} cells (open circles) was measured by nuclear fragmentation, 24 h after treatment with either perifosine (A) or edelfosine (B). Apoptosis in KB (solid squares) and KBr cells (open squares) was determined after 48 h exposure to perifosine (C) or edelfosine (D). Representative dose-reponse curves are shown, values are mean ± S.D. of quadruplicates.

edelfosine between S49 and S49^{AR} is only moderate, yet statistically significant (1.2-fold, $P < 0.05$) (compare Fig. 1A and B).

Parental KB cells were equally sensitive to perifosine and edelfosine, with EC_{50}'s of about 2 μM for both compounds. APL resistance was pronounced (>40-fold) in KBr and comparable for both perifosine and edelfosine (Fig. 1C and D).

Cells were treated with a panel of APLs to test whether resistance induced by a single APL analogue resulted in cross-resistance to other APL members. In addition to perifosine and edelfosine, cells were treated with the APL analogues miltefosine, erucylphosphocholine (ErPC) and Zentaris compound D-21805, in which the choline nitrogen atom has been replaced by arsenic. APL-induced apoptosis in S49 and KB cells was measured at different time points, 24 and 48 h, respectively, because of different apoptosis kinetics in these cells [19,29]. All tested compounds induced apoptosis in S49 at a concentration of 20 μM, edelfosine being most potent and miltefosine being only moderately effective (Fig. 2A). Interestingly, the edelfosine-resistant S49^{AR} cells showed cross-resistance to all other APLs, although the degree of resistance varied between the APLs tested (Fig. 2A). KB cells were overall highly affected by APLs, applied at 5 μM (deduced from Fig. 1), whereas the perifosine-resistant KBr cells were fully cross-resistant to apoptosis induction by the other APLs (Fig. 2B). The different degree of cross-resistance for other APLs observed for S49^{AR} and KBr suggests distinct underlying mechanisms determining their resistance.

3.2. *APL uptake depends on metabolic energy, especially in KB cells*

We next assessed the relationship between APL sensitivity and cellular uptake in S49 and KB cells. We also compared the mechanism of uptake of two APLs, perifosine and edelfosine, in these two cell types. Evidence exists that after initial insertion into the outer leaflet of the plasma membrane, APLs accumulate in lipid rafts [22,35] (see below) and undergo raft-dependent endocytosis [22,36]. To assess the possible role of endocytosis, APL uptake at 37 °C was compared with 4 °C, a temperature at which endocytosis is blocked. In S49 cells, edelfosine uptake (at 30 min) was more temperature-dependent than the uptake of perifosine (Fig. 3A). Remarkably, KB carcinoma cells showed a much higher APL uptake (more than 10-fold for perifosine) than S49 cells (at the same APL concentration, 1 μM) and the KB cellular uptake of perifosine was twice as high as edelfosine. Furthermore, APL uptake by KB cells was almost completely (94%) blocked at 4 °C (Fig. 3B). For perifosine, the temperature effect and hence the mode of cellular uptake was quite contrasting for KB and S49 cells. Perifosine uptake by S49 was only moderately (35%) sensitive to low temperature, compared to edelfosine (64%). To confirm that KB cells display a more active, temperature and energy-dependent uptake of APL, we subjected the two cell types to ATP depletion prior to APL uptake. It appeared that the APL uptake by KB cells was more compromised (55%) by such

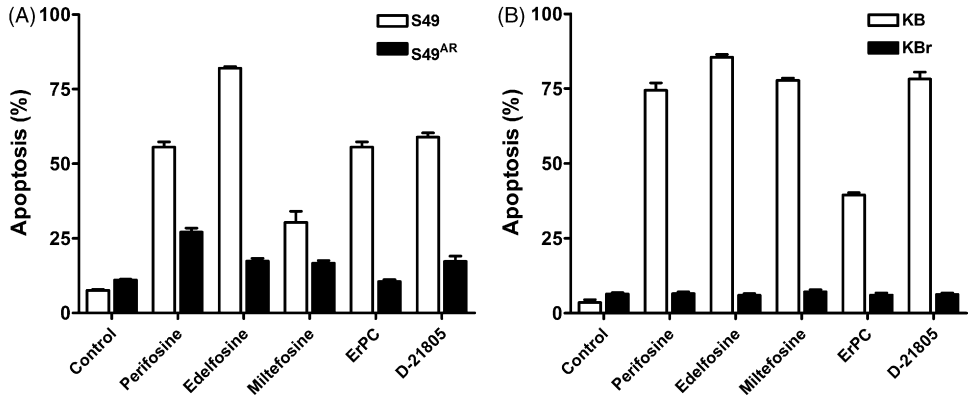

Fig. 2 – Differential sensitivity of S49 and KB cells and their APL-resistant cell variants S49^{AR} and KBr, respectively, to apoptosis induction by a panel of APL analogues indicated. Apoptosis was determined by propidium iodide staining and FACScan analysis of subdiploid nuclear fragments. (A) Apoptosis in S49 and S49^{AR} cells, 24 h after treatment with 20 μM APL. (B) Apoptosis in KB and KBr cells, 48 h after treatment with 5 μM APL. Data are means of triplicates ± S.D.

energy depletion than the S49 cells (15% for perifosine, 36% for edelfosine) (Fig. 3C).

3.3. *APLs accumulate in lipid rafts in KB and S49 cells independent of sphingomyelin content*

We have previously shown that edelfosine accumulates in lipid rafts of S49 cells [22]. We pre-incubated S49 and KB cells with radiolabeled edelfosine and perifosine, and isolated detergent-resistant lipid raft fractions from these cells. We found a similar APL distribution among the sucrose gradient fractions of both cell types. APLs accumulated in the lipid raft fractions 2–4, perifosine even more prominently (about twofold) than edelfosine (Fig. 4A and B). Furthermore, APL accumulation in lipid raft fractions was comparable between parental and resistant cells (data not shown). The bulk APLs distributing at the higher density (non-raft) fractions 7–11 is derived from all other membraneous parts (plasma and endoplasmic membranes) of the cell. We calculated that, based on protein content, perifosine and edelfosine in the combined raft fractions 2–4 were 46- and 34-fold enriched, respectively, relative to the non-raft fractions 7–11, for S49 cells. For KB cells, these enrichments were 43- and 16-fold, respectively. Our previous data [22,29] (see also Fig. 5) have revealed that, in S49 cells, only the raft-dependent routes of APL internalization are relevant for apoptosis sensitivity of these cells.

Reduced APL uptake in S49^{AR} has previously been shown to be the consequence of impaired raft-mediated endocytosis, due to downregulated SM synthase 1 expression and consequently reduced SM synthesis [22,30]. Indeed, S49^{AR} cells were deficient in SM production, as shown by the lack of [^{14}C]choline incorporation (Fig. 4C). In contrast to S49^{AR} cells, APL resistance in KBr cells was not accompanied by reduced SM synthesis. The TLC separation of radiolabeled lipid extracts from KB and KBr cells showed two comparable SM spots (Fig. 4D), typically corresponding to a SM pool with relatively short acyl chains (16C-atoms) and a SM pool with long acyl chains (predominantly C24:1) and containing a C16-dihydro-(sphinganine) species, as described previously [30]. We

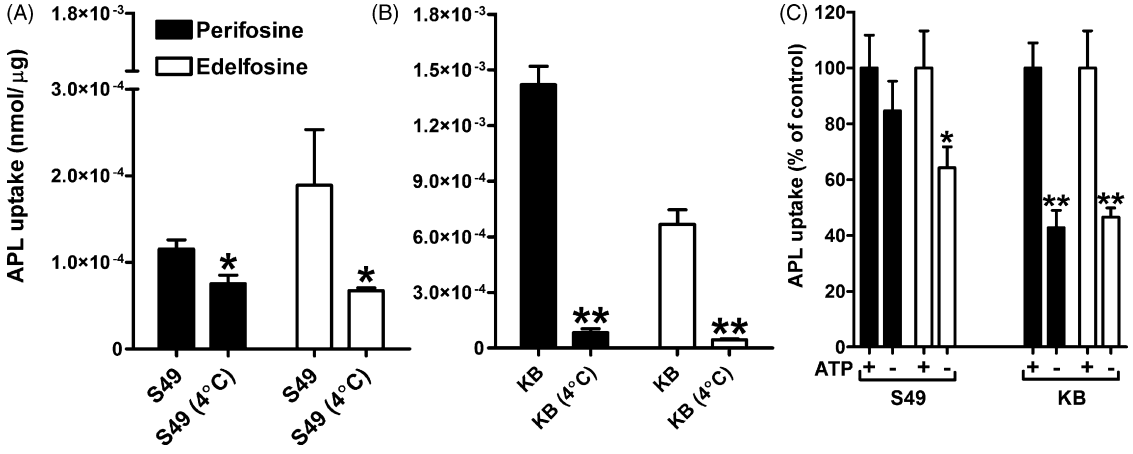

Fig. 3 – Differential uptake of edelfosine and perifosine by S49 and KB cells, depending on temperature and cellular ATP. Uptake of [^{14}C]perifosine (0.03 μCi/mL, 1 μM) and [^{3}H]edelfosine (0.15 μCi/mL, 1 μM) was determined in S49 cells (A) and KB cells (B) after 30 min incubation at 37 °C or 4 °C (indicated). (C) Effect of ATP depletion on the uptake (at 37 °C) of APL, 20 μM or 1 μM by S49 and KB cells, respectively. Values are means of triplicates ± S.D. *$P < 0.05$; **$P < 0.001$.

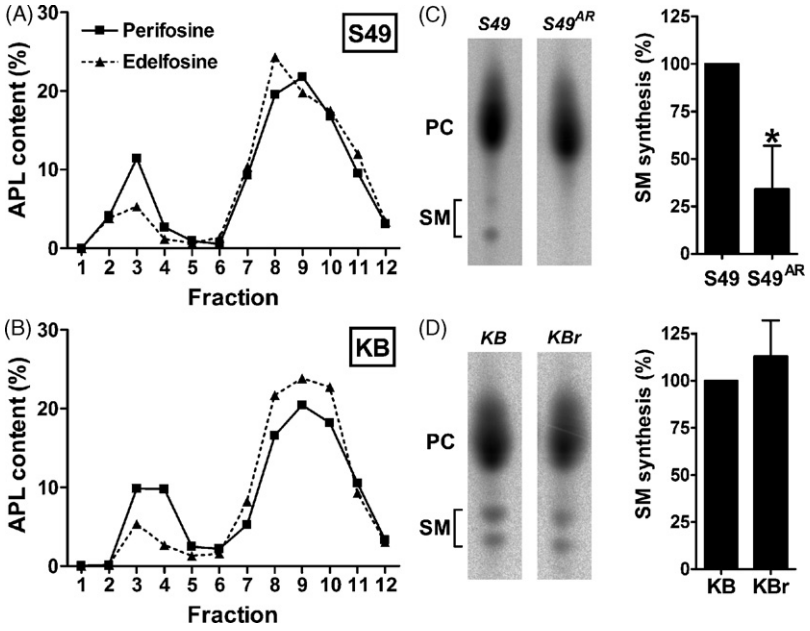

Fig. 4 – Edelfosine and perifosine accumulate in lipid rafts of S49 and KB cells, independent of sphingomyelin (SM) synthesis. Cells were treated with perifosine or edelfosine for 30 min, and rafts were subsequently isolated as described in Section 2. Fractions 2–4 represent detergent-resistant rafts. Distributions are shown after incubation with either 20 μM or 1 μM APL for S49 (A) and KB (B), respectively. Representative distributions are shown. SM synthesis is abrogated in the APL-resistant $S49^{AR}$ cells (C), but not in KBr cells (D). Cells were labeled for 8 h with [^{14}C]choline (without APL). Lipids were extracted, separated by TLC, visualized and quantified by Phosphor-Imaging (right panels; means of three experiments ± S.D.; *$P < 0.05$). PC, phosphatidylcholine; SM, sphingomyelin.

conclude that, unlike $S49^{AR}$ cells, downregulation of SM synthesis is not the mechanism of APL-resistance induction in KBr.

3.4. *APL internalization is raft-dependent in S49, but raft-independent in KB cells*

To get more insight in the mode of uptake of APLs in the two cell types, we used two inhibitors of endocytosis, methyl-β-cyclodextrin (MβCD) and chlorpromazine, that act in different ways: MβCD inhibits raft-mediated endocytosis by extracting cholesterol from the membrane, whereas chlorpromazine is commonly used as an inhibitor of clathrin-mediated endocytosis, as it inhibits clathrin-coated pit formation at the plasma membrane [37]. Cells were pretreated with either of these inhibitors, followed by treatment (for 1 h) with equi-effective concentrations of APLs (20 μM for S49, 1 μM for KB). The effect of these inhibitors on the relative APL uptake by the two cell

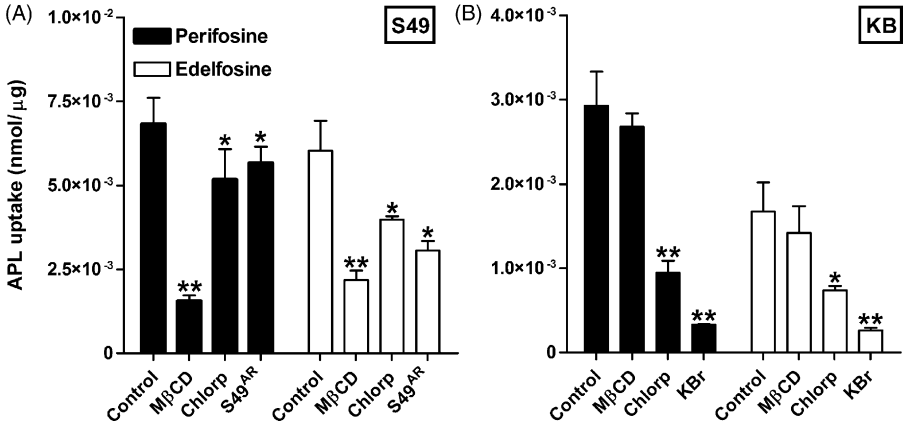

Fig. 5 – Cell type-dependent effect of pharmacological inhibitors of endocytosis on the uptake of APLs. Cells were pretreated for 30 min with methyl-β-cyclodextrin (MβCD; 2.5 mg/mL) (blocks raft-dependent endocytosis) or chlorpromazine (20 μM) (blocks raft-independent endocytosis), and subsequently incubated for 1 h with 0.03 μCi [^{14}C]perifosine or 0.15 μCi [^{3}H]edelfosine at 20 μM for S49/$S49^{AR}$ cells (A) and at 1 μM for KB/KBr cells (B). Uptake values are expressed in nmol APL/μg cellular protein, as means of triplicates ± S.D. *$P < 0.05$; **$P < 0.001$.

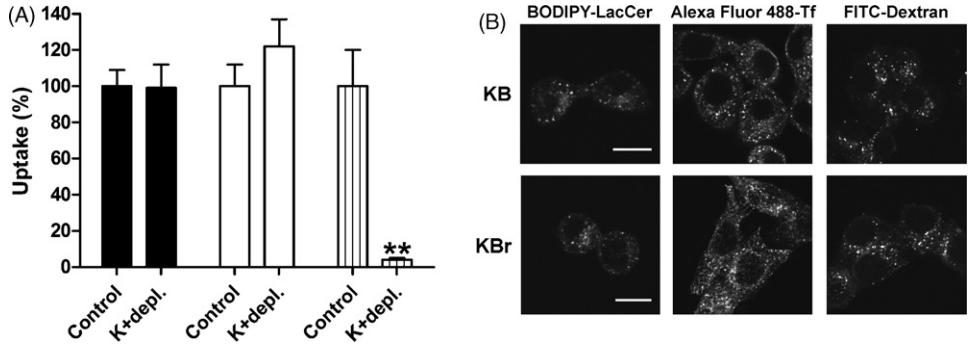

Fig. 6 – No defects in canonical endocytosis routes can explain abrogated APL uptake in KBr cells. (A) APL uptake in KB cells is not affected by K^+ depletion (which blocks clathrin-mediated endocytosis). Cells were left untreated or were K^+ depleted, as described in Section 2. Uptake of [^{14}C]perifosine (0.03 μCi, 1 μM) (open bars), or [^{3}H]edelfosine (0.15 μCi, 1 μM) (closed bars), or 1 ng [^{125}I]EGF (684 mCi/mmol) (positive control; hatched bars) was measured at 30 min. Values represent percentage uptake relative to controls (mean of triplicates ± S.D. of a representative experiment; $^{**}P < 0.001$. (B) Confocal microscopy of fluorescently labeled markers of endocytosis. KB and KBr cells, grown on cover slips, were incubated with BODIPY-lactosylceramide (5 μM) (marker for raft-mediated endocytosis), Alexa-488-labeled transferrin (10 μg/mL) (marker for clathrin-mediated endocytosis) or FITC-dextran (2 μg/mL) (marker for fluid-phase endocytosis) for 30 min at 37 °C. Uptake of these fluorescent markers by KB cells (upper panels) and KBr cells (lower panels) was visualized by confocal microscopy. The bar in the figure represents 20 μm.

types was very different (Fig. 5). MβCD reduced the uptake of edelfosine and perifosine in S49 cells by as much as 64% and 77%, respectively, but only by 15% and 8%, respectively, in KB cells. Chlorpromazine, on the other hand, more effectively reduced the edelfosine and perifosine uptake by KB cells (56% and 68%, respectively) than by S49 cells (34% and 24%, respectively).

When we compare cellular uptake of edelfosine with perifosine, we find relatively little difference in perifosine uptake between S49 and S49^{AR} cells, in agreement with Fig. 1A. Although absolute edelfosine uptake by KB cells was lower than perifosine uptake, the effects of MβCD and chlorpromazine on cellular uptake were similar for the two APLs (Fig. 5B). Neither of the two endocytosis inhibitors reduced the APL uptake to the level of the APL-resistant KBr clone, which displayed a ninefold reduced perifosine uptake. Although the chlorpromazine effect on APL uptake would suggest the involvement of clathrin-mediated endocytosis [37], we did not find in KBr cells a defect in the clathrin-dependent internalization of fluorescently labeled-transferrin by its cognate receptor (Fig. 6B). Moreover, we found that another established way to block clathrin-mediated endocytosis, by K^+ depletion of cells, failed to reduce the uptake of both edelfosine and perifosine by KB cells, whereas, as a positive control, the uptake of ^{125}I-EGF via the epidermal growth factor receptor (EGFR) was fully blocked (Fig. 6A). Therefore, we do not believe that the clathrin-dependent pathway is a major route of APL endocytosis in KB cells. We also found no differences between KB and KBr cells in the raft-mediated endocytosis of a fluorescent raft marker, BODIPY-lactosylceramide [38] and the uptake of fluorescent dextran via fluid-phase endocytosis (Fig. 6B). In conclusion, we found no clear defect in three different routes of endocytosis that would explain the resistance of KBr cells to APLs.

To address the mechanism of internalization of APL-like compounds further, we studied the uptake of exogenous LysoPC. From our previous work in HeLa cells [36], we know that exogenous LysoPC does not accumulate in lipid rafts and is internalized independently from these microdomains, by transbilayer flipping, at least to a significant extent. Immediately after membrane traversal, LysoPC is fully acylated to PC [36]. We therefore incubated KB and KBr cells with [^{14}C]LysoPC and followed in time the production of [^{14}C]PC (Fig. 7). From a comparison of the absolute (arbitrary) units of [^{14}C]PC synthesized in time (Fig. 7A) with the [^{14}C]LysoPC left in the cells (Fig. 7B), it follows that, indeed, most of the LPC is rapidly (within a few minutes) converted into PC. This is even more clear when expressed in percentages conversion of LysoPC into PC (Fig. 7C). Furthermore, similar to edelfosine and perifosine, the LysoPC internalization and subsequent conversion to PC was much decreased in the KBr cells (Fig. 7A). The residual LysoPC in KBr cells was higher than in KB cells (Fig. 7B), indicative of impaired internalization in the KBr cells. As a control, LysoPC to PC conversion in KB and KBr cell lysates were not different (data not shown), indicating that the intracellular acyl-transferase activities were similar in these cells, and that the reduced LysoPC to PC conversion in intact KBr cells was indeed the consequence of a blockade in transbilayer movement of the LysoPC. Similar to APL uptake, this LysoPC internalization was sensitive to chlorpromazine treatment (Fig. 7A).

Collectively, these results suggest that, contrary to S49 cells, APL uptake in KB cells is mainly raft-independent, but dependent on a yet undefined energy-dependent (active) uptake mechanism that can be inhibited by chlorpromazine. We find no clear evidence for a defective endocytosis in APL-resistant KBr cells. Since these cells also display impaired uptake of natural LysoPC, we rather think of a defect in transmembrane flipping at the level of an undefined lipid transporter.

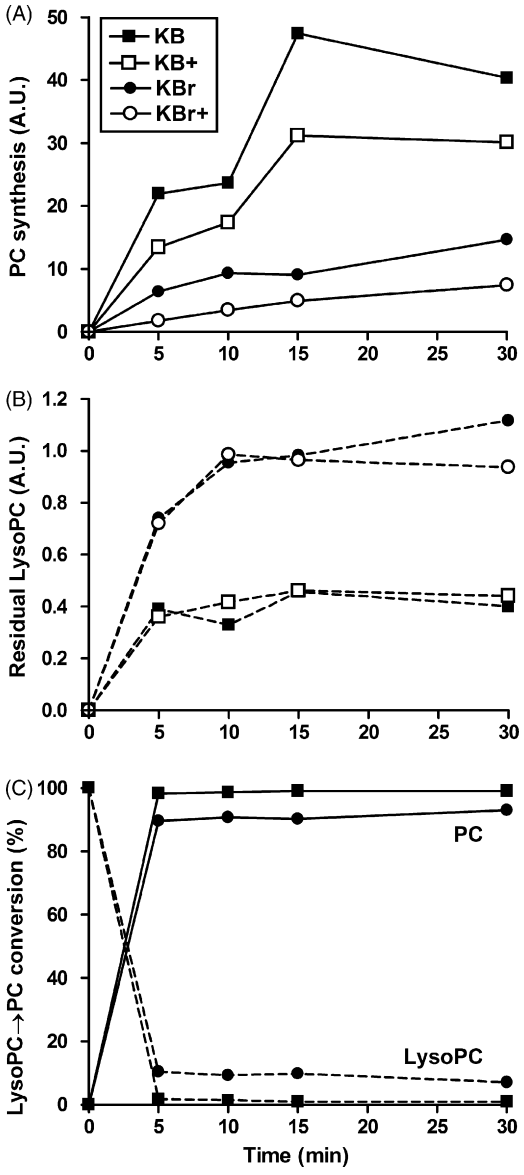

Fig. 7 – Internalization of exogenous LysoPC and subsequent acylation to PC in KB and KBr cells. KB cells (squares) and KBr cells (circles) were incubated for indicated time periods with 0.025 μCi [^{14}C]LysoPC, in the absence (solid symbols) or presence of chlorpromazine (20 μM; 30 min pre-incubation) (KB+, KBr+; open symbols). Cells were then washed three times with PBS. Lipids were extracted and separated by TLC. [^{14}C]PC (panel A, solid lines) and residual [^{14}C]LysoPC (panel B, broken lines) were quantified by Phosphor-Imaging. Values represent arbitrary units (AU) of a representative experiment. (C) Percentage of conversion of [^{14}C]LysoPC (broken lines) into [^{14}C]PC (solid lines) as a function of time.

4. Discussion

In this study, we show that the cellular uptake and consequent toxicity of anti-cancer APLs in the KB squamous cell carcinoma differs significantly from S49 lymphoma cells. For this latter cell type, we previously described in much detail how the APL edelfosine is internalized by clathrin-independent, raft-mediated endocytosis [22,30]. The present study suggests that, in S49 cells, the other ALPs are also taken up by this route. We showed for example that the uptake of edelfosine and perifosine in these cells was inhibited by the cholesterol-chelating agent MβCD, which disrupts lipid rafts. KB cells, on the other hand, were much more sensitive to APLs than S49 cells due to a higher cellular uptake. Contrary to S49 cells, this high APL uptake in KB cells was mainly raft-independent but, instead, more dependent on ambient temperature and metabolic energy. In addition, APL uptake in KB cells was inhibited by chlorpromazine, which is usually suggestive of a clathrin-dependent uptake [37] but, relevant to our present study, may have relatively non-specific phospholipid ''translocase'' effects as well [39,40]. However, K^+ depletion, another way to block clathrin-dependent endocytosis [37], had little effect on APL uptake in KB cells yet, prevented internalization of ^{125}I-EGF by its cognate receptor via the clathrin pathway. Moreover, APL-resistant KBr cells show dramatically impaired uptake of (radiolabeled) edelfosine, perifosine and even natural LysoPC, while their uptake of transferrin, presumably via clathrin-dependent receptor internalization was unaffected. So, there is much evidence against clathrin-dependent endocytosis of APL as a major route to induce toxicity in KB cells. In this regard, there is a remarkable resemblance of the KB/KBr system with the macrophage-like RAW cells and their APL-resistant counterparts, described previously [18].

The crucial role of lipid rafts in the uptake of edelfosine by S49 lymphoma cells [22] was recently further explained. We found that the edelfosine-resistant cell variant S49^{AR} was deficient in the raft constituent sphingomyelin (SM) due to downregulated expression of SM synthase 1. This inhibition of SM synthesis in the *trans*-golgi network abrogated lipid raft vesicular trafficking/recycling and raft-dependent endocytosis of edelfosine and apoptosis induction in these cells [30]. We have now demonstrated that these SM-deficient S49^{AR} cells are not only resistant to edelfosine but also to the other APLs. Interestingly, we found a similar APL cross-resistance in the KBr cells. While parental KB carcinoma cells take up high amounts of APLs, such as miltefosine [31] and perifosine [19] (also in this study), the KBr variant cells, originally made resistant to perifosine, were even stronger cross-resistant to APLs than S49^{AR} cells. Importantly however, this resistance was based on a different mechanism. Contrary to S49^{AR} cells, the KBr cells display normal SM synthesis, suggesting that APL resistance was independent on lipid rafts. This notion is supported by the different effects in these cells of MβCD, which sequesters cholesterol and inhibits raft-dependent endocytosis. We found that this lipid raft-disrupting agent dramatically reduced APL uptake in S49 cells, but not in KB cells.

Targeting of lipid rafts was recently suggested to underlie the selective induction of apoptosis in multiple myeloma cells by both edelfosine and perifosine [41]. Following this concept, Mollinedo et al. reported that edelfosine was localized in rafts in leukemic cells, in agreement with our data, but not in solid tumor cells [35]. We found some APL accumulation in isolated lipid rafts of the solid tumor cell KB, but without an apparent function there. Thus it seems that APL incorporation in lipid

rafts and the role of these rafts in APL-induced cytotoxicity is very much dependent on the cell type.

The group of Berkovic and Fleer have made KB cells resistant to miltefosine (hexadecylphosphocholine) (yielding a distinct KBr cell variant) by prolonged culturing in the presence of this drug [21], in a similar way as the $S49^{AR}$ cells were made resistant to edelfosine. Their KBr cells contained even more SM than the parental KB cells, while the uptake of miltefosine was reduced to 20–40%. These cells were cross-resistant to edelfosine and ilmofosine (another APL) and showed reduced uptake of inositol, for reasons that remain unclear. The KBr cells used in our studies were obtained via a process that included mutagenesis. This method possibly induced genetic defects related to energy-mediated uptake processes, since active, energy-dependent APL uptake was completely blocked in these KBr cells, whereas SM synthesis and uptake of the raft marker lactosylceramide was not impaired. Taken together, our results and those of the German group [21,31] strongly argue against a role for SM synthesis and lipid rafts, but suggest a defect in energy-dependent active uptake to explain the APL resistance in KBr carcinoma cells.

While no apparent defects in SM synthesis, lipid raft- or clathrin-dependent, or fluid-phase endocytosis are detectable in KBr cells to explain their resistance to APLs, what other energy-dependent uptake mechanism could be involved in these cells? We found that APL uptake in KB cells was inhibited by chlorpromazine (Fig. 5) and by cytochalasin B (data not shown), which disrupts actin filaments. Both drugs can inhibit endocytosis, and although the mentioned canonical routes of endocytosis seem not to be affected in the KBr cells, it remains possible that APLs are taken up by an as yet poorly defined endocytic pathway in KB cells. Alternatively, the active APL uptake by KB cells could be mediated by an unknown ATP-driven transporter (see below) that may somehow be compromised by chlorpromazine [39,40] and cytochalasin B-induced cytoskeleton disruption.

In two non-mammalian systems, there is evidence for the involvement of a P-type transmembrane ATPase that actively translocates APL molecules like miltefosine and perifosine over the plasma membrane. First, in the parasite *Leishmaniana*, resistance to miltefosine has been attributed to inactivation of the transporter protein LdMT and its beta-subunit LdRos3 [42,43]. Secondly, in yeast there are similar P-type ATPases, Dnf1p and Dnf2p that, in association with their non-catalytic beta-subunit Lem3p, act as ''flippases'' for inward transbilayer movement of aminophospholipids, lysophospholipids and APLs [44–46]. It is possible that mammalian counterparts of these ATPases exist that can act as a flippase for APL uptake. A recent study in Caco-2 intestinal epithelial cells indeed suggests the existence of a yet undefined carrier-mediated uptake of miltefosine [47]. Whether such a flippase exists in KB cells and is inactivated in KBr cells remains to be investigated.

In conclusion, we report a critical role of raft-mediated endocytosis in the uptake of APLs by S49 lymphoma cells, whereas uptake by KB carcinoma cells occurs mainly by a raft-independent, energy-dependent route that is sensitive to chlorpromazine and does not involve the canonical endocytic pathways. Individual APLs are taken up to different extent, which correlates with cell sensitivity towards the drug. The precise mode of APL internalization by KB cells and the mechanism of resistance in KBr cells needs to be further defined with the help of additional specific markers for endocytic activity or the possible identification of an APL transmembrane transporter.

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