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The anti-tumor alkylphospholipid perifosine is internalized by an ATP-dependent translocase activity across the plasma membrane of human KB carcinoma cells

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Abstract

Perifosine is a promising anticancer alkylphospholipid (ALP) that induces apoptosis in tumor cells. Here we report evidences against a role of endocytosis in perifosine uptake by human KB carcinoma cells. We have generated a KB cell line resistant to perifosine (KB PER^R clone10), which shows cross-resistance to the ALPs miltefosine and edelfosine, a marked impairment in the uptake of ^{14}C-perifosine at both 37 °C and 4 °C, and no signs for active efflux of the drug. KB PER^R clone10 cells show a similar rate of raft-dependent endocytosis with respect to the parental cells, and silencing of both clathrin and dynamin in the latter causes only minor changes in the rate of perifosine uptake. Perifosine uptake is a temperature- and ATP-dependent, *N*-ethylmaleimide- and orthovanadate-sensitive process in parental cells. Accumulation of ^{14}C-perifosine and the fluorescent phospholipid analogue 6-[(7-nitrobenz-2-oxa-1,3-diazol-4-yl)aminocaproyl]-phosphatidylethanolamine (NBD-PE) is inhibited by perifosine in a concentration-dependent manner in parental cells. Moreover, NBD-PE accumulation is slower in PER^R clone10 cells and correlated with phosphatidylserine exposure in their plasma membrane surface. Together, all these data suggest a role of plasma membrane translocation by a putative phospholipid translocase, rather than endocytosis, as the true mechanism for ALPs uptake in KB carcinoma cells.

Keywords: Alkylphospholipids; Perifosine uptake; Tumor cells; Endocytosis; Aminophospholipid translocase

1. Introduction

The synthetic antineoplastic 1-*O*-octadecyl-2-*O*-methyl-*rac*-glycero-3-phosphocholine (ET-18-OCH_3, edelfosine), and hexadecylphosphocholine (HePC, miltefosine), constitute a class of anticancer drugs that act at the level of cell membranes and, therefore, their effects are independent of the proliferative state of target cells [1,2]. Edelfosine has become the prototype of the synthetic ALPs, and selectively induces apoptosis in both human tumor cell lines and primary tumor cell cultures from cancer patients [3]. Its mechanism of apoptosis induction consists of interfering with phospholipid turnover [4,5], causing persistent activation of c-Jun amino-terminal kinase pathway (JNK) [6] and mitochondrial-related events [7,8], and the activation of the death receptor Fas/CD95 independently of its ligand FasL, through its co-clustering with membrane rafts [9,10].

Perifosine (octadecyl-[*N*,*N*-dimethyl-piperidinio-4-yl]-phosphate) is an oral bioactive novel ALP, structurally related to miltefosine but with an improved tolerability profile, that has been reported to promote apoptosis in a similar manner like edelfosine: by recruiting death receptors and downstream signalling molecules into lipid rafts [11]. Perifosine displays a strong antineoplastic effect in human tumor cell lines [12] and is currently being tested in phase II clinical trials for treatment of major human cancers. However, the action of the ALPs is very specific and dependent on the malignant state of the tumor cells, existing a clear correlation between cellular uptake and ALP-induced apoptosis [3]. Therefore, uptake of ALPs by tumor cells is a prerequisite for triggering the intracellular events that in the last

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term cause cell death, and a threshold for intracellular ALP concentration must be reached in order to induce apoptosis [9].

Previous reports suggest that ALPs are internalized via raft-mediated endocytosis in both mouse lymphoma [13] and human carcinoma [14] cells. However, this mechanism of internalization cannot be universal, since other works have ruled out endocytosis to play a role in ALP uptake in other mammalian cell lines [15–17]. Instead, they have postulated the existence of a "cellular structure", putatively absent or present in low basal amounts in normal cells but synthesized in higher amounts in tumor cells, as responsible for the ALP uptake and the subsequent ALP-induced apoptosis [3]. In fact, Zoeller et al. [17] have generated cell mutants derived from the edelfosine-sensitive murine RAW 264.7 cells that showed a marked impairment in their ability to accumulate edelfosine and both choline and ethanolamine phospholipids, with normal rates of endocytosis. This suggests that the postulated "cellular structure" responsible for ALP uptake could be a putative phospholipid translocase in the plasma membrane, at least in some tumor types.

In the present work we provide evidences that strongly support membrane translocation by an ATP-dependent, *N*-ethylmaleimide- and vanadate-sensitive phospholipid translocase as the most plausible mechanism for ALPs uptake in human KB epidermal carcinoma cells, and reject a main role of endocytosis in the uptake of ALPs in these tumor cells.

2. Materials and methods

2.1. Chemicals

Miltefosine (hexadecylphosphocholine), perifosine (octadecyl-[*N*,*N*-dimethyl-piperidinio-4-yl]-phosphate), and [^{14}C]-perifosine (30.9 mCi/mmol) were provided by Zentaris GmbH (Frankfurt, Germany). Edelfosine (1-*O*-octadecyl-2-*O*-methyl-*rac*-glycero-3-phosphocholine), anti-clathrin and anti-dynamin antibodies were from Calbiochem. BODIPY-lactosylceramide, Alexa Fluor 488-Annexin V, Alexa Fluor 488-transferrin and Alexa Fluor 488-labeled Dextran 10,000 were from Invitrogen. Pre-designed ON-TARGETplus siRNA for human dynamin mRNA was from Dharmacon. (7-nitro-2-1,3-benzoxadiazol-4-yl)-labeled phospholipids (NBD-phospholipids) were from Avanti Polar Lipids. GF120918 was kindly provided by GlaxoSmithKline. All other chemicals were from Sigma-Aldrich.

2.2. Cell cultures

The human epidermal carcinoma KB cell line was obtained from the American Type of Cell Cultures (ATCC number CCL-17), and cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% (v/v) fetal bovine serum (FBS), 2 mM Glutamax I, and 250 U/ml penicillin G plus 250 μg/ml streptomycin sulphate (Invitrogen), at 37 °C in a humidified atmosphere and 5% CO_2. In order to generate a perifosine-resistant cell line, parental KB cells were subjected to mutagenesis with ethyl-methanesulphonate (1 mg/ml) for 4 h, and then extensively washed and grown in fresh medium for 48 h. After that, they were subjected to several selective rounds of incubation with high concentrations of perifosine (IC90) for short intervals (2–3 h), and the surviving cells of each round were allowed to expand before a new incubation in the presence of the drug. After this selection process, the resulting survival population was able to grow in the presence of 20 μM perifosine indefinitely, and the resistant phenotype was stable for more than 1 month growing without drug pressure. Afterwards, this resistant population was plated at low density and single colonies were isolated, expanded, and its inability to accumulate ^{14}C-perifosine analyzed. In this way, we were able to isolate several resistant clones, being the clone10 (KB PER^R clone10) the one that showed the lowest levels of perifosine accumulation of all the tested clones. Considering the stability of the resistant phenotype, it was unnecessary to maintain the PER^R clone10 in the continuous presence of perifosine: instead, we routinely grew them for 1–2 passages with 20 μM perifosine every month.

2.3. MTT survival assays

Sensitivity of parental KB (Wt) and KB PER^R clone10 cells to ALPs was determined by the MTT colorimetric assay as described [18]. Briefly, cells were plated in 96-well plates at a density of 1×10^4 cells (100 μl/well), allowed to attach overnight, and ALPs were added in serial dilutions. After 24 h, cells were incubated with 0.5 mg/ml MTT solution for 4 h, and the resulting formazan crystals dissolved by adding 50 μl 10% SDS overnight. Absorbance was measured at 540 nm with a VERSAmax microplate reader (Molecular Devices). Dose–response curves were generated by plotting percentage of cell survival against concentration of ALPs, and the data points (mean±S.D.; $P<0.05$, $n=3$) were fitted by non-linear regression to a four parameter logistic curve using SigmaPlot 2000 for Windows (SPSS Inc.) to determine IC50 values.

2.4. ^{14}C-perifosine uptake

Cells were plated in 24-well plates (2.5×10^4 cells/well), and incubated overnight. Afterwards, cells were pre-treated for 30 min with different drugs and inhibitors dissolved in DMEM+10% FBS, except indicated otherwise: 10 μM NBD-PE, 2 mM *N*-ethylmaleimide (NEM), 5 mM sodium orthovanadate, ATP-depleting medium [HPMI buffer (100 mM NaCl, 5.4 mM KCl, 0.04 mM $CaCl_2$, 10 mM Hepes, 25 mM $NaHCO_3$) with 20 mM 2-deoxy-D-glucose and 10 mM sodium azide], 10 μM cyclosporine A, 3 μM GF120918, increasing concentrations of methyl-β-cyclodextrin (MβCD) up to 10 mg/ml in DMEM without FBS, or chlorpromazine, perifosine, edelfosine and miltefosine up to 20 μM (at both 37°C and 4 °C), where indicated. Hereafter, cells were incubated with ^{14}C-perifosine (0.03 μCi/ml, 1 μM) at either 37 °C or 4 °C for 1–2 h or 20–50 min, respectively, depending on the experiment, and subjected to back-exchange using 2% fatty acid-free BSA in HPMI for 5 min at 4 °C, in order to remove the excess of perifosine in the external face of the plasma membrane. In the case of samples pre-treated with MβCD, the cells were thoroughly washed before adding ^{14}C-perifosine in order to completely remove MβCD, since we observed that the cholesterol-depleting agent also sequestered perifosine and hence lowered the concentration of free ^{14}C-perifosine. We also checked under the microscope the cells immediately after chlorpromazine or MβCD pre-treatment, and they look healthy, with no observable changes in cell morphology or membrane integrity. We observed, however, some cell detachment when incubated with the highest concentration of MβCD employed (10 mg/ml); however, the cell loss was took into account when calculating the accumulation of ^{14}C-perifosine (always normalized to protein content), it was never higher than 20% of total cell number, and the remaining, attached cells (those that really accumulated ^{14}C-perifosine) looked perfectly normal. For efflux experiments, KB Wt and PER^R clone10 cells were incubated with ^{14}C-perifosine for 15 min or 5 h, respectively, in order to achieve similar intracellular concentrations of the drug in both cell lines at time zero of efflux. After this loading period, the cells were washed and incubated in fresh, drug-free culture medium, allowing them to extrude the radioactive drug analogue. At appropriate time points, cells were washed once with ice-cold HPMI buffer, followed by back-exchange with 2% fatty acid-free BSA in HPMI for 5 min at 4 °C, washed once more with HPMI buffer, and subsequently lysed in 1% Triton X-100 plus 0.25 N NaOH. The cellular lysated was further diluted in liquid scintillation cocktail (EcoLume™, MP Biomedicals) for counting in a LKB/WALLAC 1219 Rack-Beta Scintillation Counter. The radioactivity values obtained, expressed in counts per minute (cpm), was normalized for the protein content of the samples.

2.5. Accumulation of fluorescent phospholipid analogues

Cells previously seeded in 24-well plates the day before, were incubated for either 15 min at 37 °C or 40 min at 4 °C with 2 μM NBD-phosphatidylethanolamine, -phosphatidylserine or -phosphatidylcholine (NBD-PE, -PS, -PC) diluted in DMEM+10% FBS. For competition assays, we used 0.2 μM NBD-

PE and co-incubated with increasing concentrations of perifosine (up to 10 μM). Chlorpromazine (up to 20 μM) or MβCD (up to 10 mg/ml) was used as inhibitor of NBD-PE uptake. After the incubation times, the plates were placed on ice and all subsequent steps were performed at 4 °C: washed once with HPMI buffer, back-exchanged with 2% fatty acid-free BSA in HPMI for 5 min at 4 °C, washed once more with phosphate-buffer saline (PBS), trypsinized for 1 min and resuspended in PBS+5 mM EDTA. The fluorescence of the samples was immediately measured by using FACSCalibur cytometer (BD Biosciences), and the data were analyzed using the BD CellQuest™ Pro software.

2.6. Alexa Fluor 488-Annexin V labeling of KB Wt and KB PER^R clone10 cells

We used an adaptation of the protocol described previously in [19] for labeling adherent cells with Annexin V. Briefly, we labeled cell cultures in exponential growth before harvesting, with 1 μg/ml Alexa Fluor 488-Annexin V in DMEM medium without FBS for 1 h at 4 °C. We did so in order to avoid the overestimation of phosphatidylserine exposure due to damages caused to cell membranes during the harvesting process. Then, cells were rinsed twice with ice-cold DMEM and resuspended in 0.5 ml DMEM by gently aspirating with a pipette. Finally, propidium iodide (1 μg/ml final concentration) was added and the cells kept on ice 5 min before immediate analysis by flow cytometry. PI-positive cells were excluded from the analysis.

2.7. Measurement of endocytosis rates

Cells plated in 24-well plates the day before were incubated with Alexa-488 fluorescently labeled human transferrin (25 μg/ml) in serum-free medium, BODIPY-lactosylceramide (0.5 μM) or with the fluorescent fluid-phase marker Alexa Fluor 488-Dextran 10,000 (0.25 mg/ml) in complete medium for 7 min at 37 °C. The cells to be labeled with the fluorescent endocytic markers transferrin or BODIPY-lactosylceramide were previously treated as follows: the former was serum-starved for 30 min at 37 °C before adding the fluorescently labeled transferrin; the latter was incubated 15 min with BODIPY-lactosylceramide at 4 °C, to allow the partitioning of the fluorescent marker into the cholesterol-enriched micro-domains of the plasma membrane, then thoroughly washed with ice-cold PBS to remove the excess of fluorophore, and warmed up to 37 °C for 7 min. Endocytosis was stopped on ice. Cells were washed thoroughly with cold PBS to remove the excess of untaken fluorescence, back-exchanged twice with 2% defatted BSA at 4 °C 10 min each in the case of the cells labeled with BODIPY-lactosylceramide, or acid-stripped 30 s at 10 °C with HPMI, pH 3.5 in the case of those labeled with Alexa Fluor 488-transferrin to remove the receptor-bound transferrin analogue [20]. Then, cells were trypsinized and resuspended in cold PBS+0.5 mM EDTA. The samples were immediately analyzed by flow cytometry using a FACSCalibur cytometer (BD Biosciences), and the data were analyzed using the BD CellQuest™ Pro software.

2.8. siRNA-based dynamin knockdown

Human dynamin 1, a key protein in both clathrin- and raft-dependent endocytosis, was targeted using SMARTpool siRNA oligonucleotides (Dharmacon). siRNA oligonucleotides (100 nM) were transfected into cells seeded the day before on 24-well plates (30–50% confluent) with 1:500 Dharmafect I (Dharmacon) in Opti-MEM reduced-serum medium (Invitrogen) for 5 h. Then, the transfection mixture was replaced by fresh DMEM+10% FBS, and the cells were further cultured for 48 h before the experiments were performed.

2.9. Western blotting

After 48 h of siRNA treatment, cells were washed twice with cold PBS, lysed by the addition of lysis buffer (5 M urea, 1% SDS, 10 mM Tris pH 6.8, 0.1 mM EDTA, 1% β-mercapto-ethanol, plus 1:50 cocktail of protease

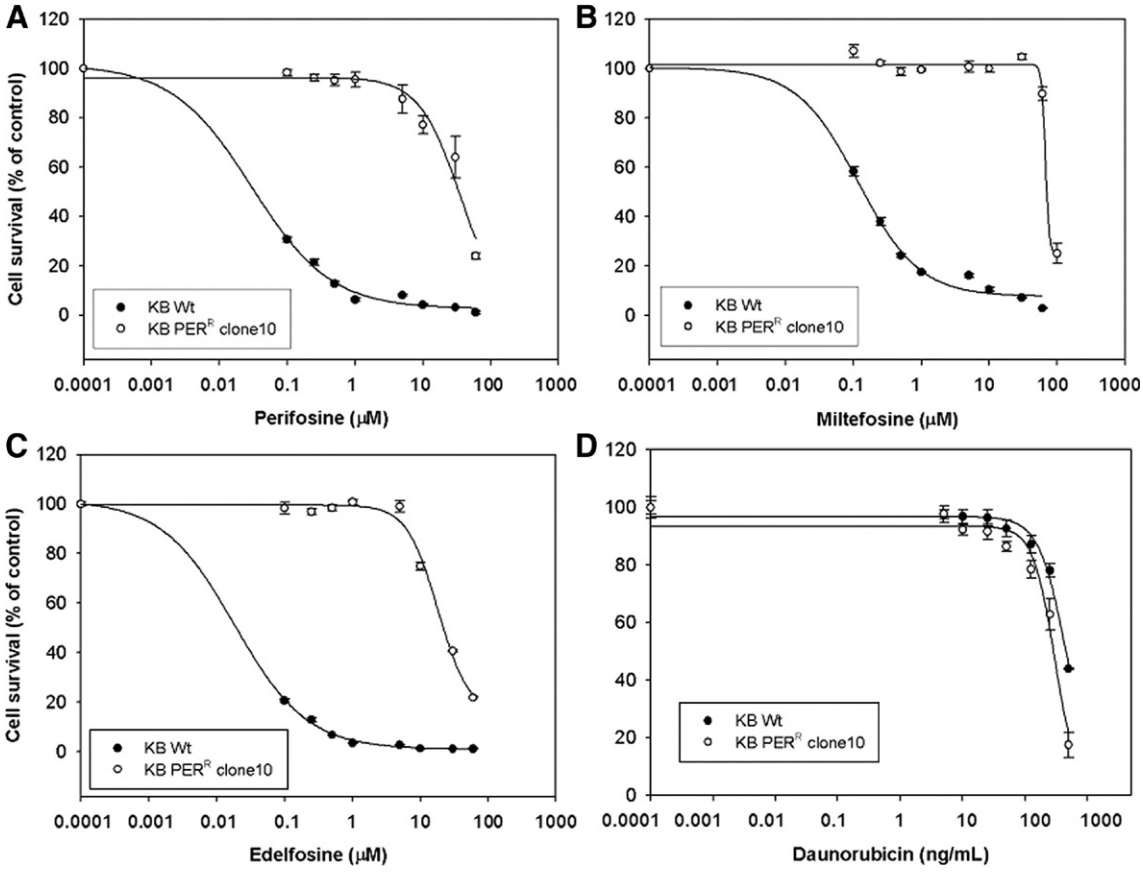

Fig. 1. Sensitivity of KB Wt and KB PER^R clone10 cells to several cytotoxic drugs: A) perifosine; B) miltefosine; C) edelfosine; D) daunorubicin. There have been depicted representative experiments of three different experiments performed in triplicate for each drug concentration.

inhibitors from Sigma) subsequent to cell suspension in an equal volume of PBS 0.1% Triton X-100 and incubated at −20 °C for 15 min. The samples (25 μg protein per lane) were resolved by 10% SDS-PAGE and transferred to PVDF membranes. Membranes were subsequently blocked for 1 h at room temperature in 5% defatted milk in PBS 0.05% Tween 20 (PBS-T), and washed three times with PBS-T. Immunodetection was performed by incubating the blocked membranes with monoclonal primary antibodies at room temperature for 1 h, followed by several washes and subsequent incubation with horseradish peroxidase-conjugated anti-mouse IgG (Promega). Immunoreactive proteins were visualized using a chemiluminescence detection kit (Pierce ECL Western Blotting Substrate) according to the manufacturer's instructions. To strip and reprobe, the membranes were incubated in Restore Western Blot Stripping Buffer (Pierce) at 37 °C for 15 min, washed, blocked, and immunoblotted with monoclonal anti-α-tubulin antibodies (Sigma) as protein loading control.

3. Results

3.1. KB PER^R clone10 cells are cross-resistant to several ALPs, but not to classical DNA-targeting anti-cancer drugs

We have generated a cell clone highly resistant to perifosine (KB PER^R clone10) from the parental human KB (Wt) cells by mutagenesis and several rounds of selection at increasing concentrations of perifosine. We performed MTT-based colorimetric survival assays and found that the PER^R clone10 displayed a high resistance to perifosine (IC50 = 35.8 ± 3.5 μM) with respect to KB Wt cells (IC50 < 0.1 μM) (Fig. 1A). Moreover, KB PER^R clone10 cells showed also cross-resistance to miltefosine (Fig. 1B) and edelfosine (Fig. 1C) compared with KB Wt cells (for PER^R clone10 and Wt cells, respectively: miltefosine IC50s = 66.7 ± 7.8 vs. 0.1 ± 0.02 μM; edelfosine IC50s = 18.1 ± 3.4 vs. < 0.1 μM).

On the other hand, we wanted to test if KB PER^R clone10 cells were also resistant to other classical anti-cancer drugs like the anthracycline daunorubicin, and found that KB Wt and PER^R clone10 cells were equally sensitive to this drug (Fig. 1D). All these findings suggest that: first, whatever the mechanism of perifosine resistance in KB PER^R clone10 cells, it should be common for all ALPs tested; second, this mechanism does not account for resistance to classical anti-cancer drugs; and third, at least those elements shared by both daunorubicin- and ALPs-triggered cell death pathways (Bid, Bax, Bcl-2, cytochrome c release, and activation of the effector caspase-8 and caspase-3) [8,21] should also be functional in KB PER^R clone10 cells as well as in Wt cells. Therefore, abrogation of cell death pathways cannot account for the ALPs-resistance phenotype observed in these cells.

3.2. KB PER^R clone10 cells accumulate much lower levels of ^{14}C-perifosine than parental cells due to a defect in perifosine internalization

We performed ^{14}C-perifosine accumulation experiments in KB Wt and PER^R clone10 cells, in order to investigate their respective abilities to internalize the drug, and found that the rate of radiolabelled drug uptake at 37 °C was much slower in clone 10 than in Wt cells (Fig. 2A). To establish whether the observed

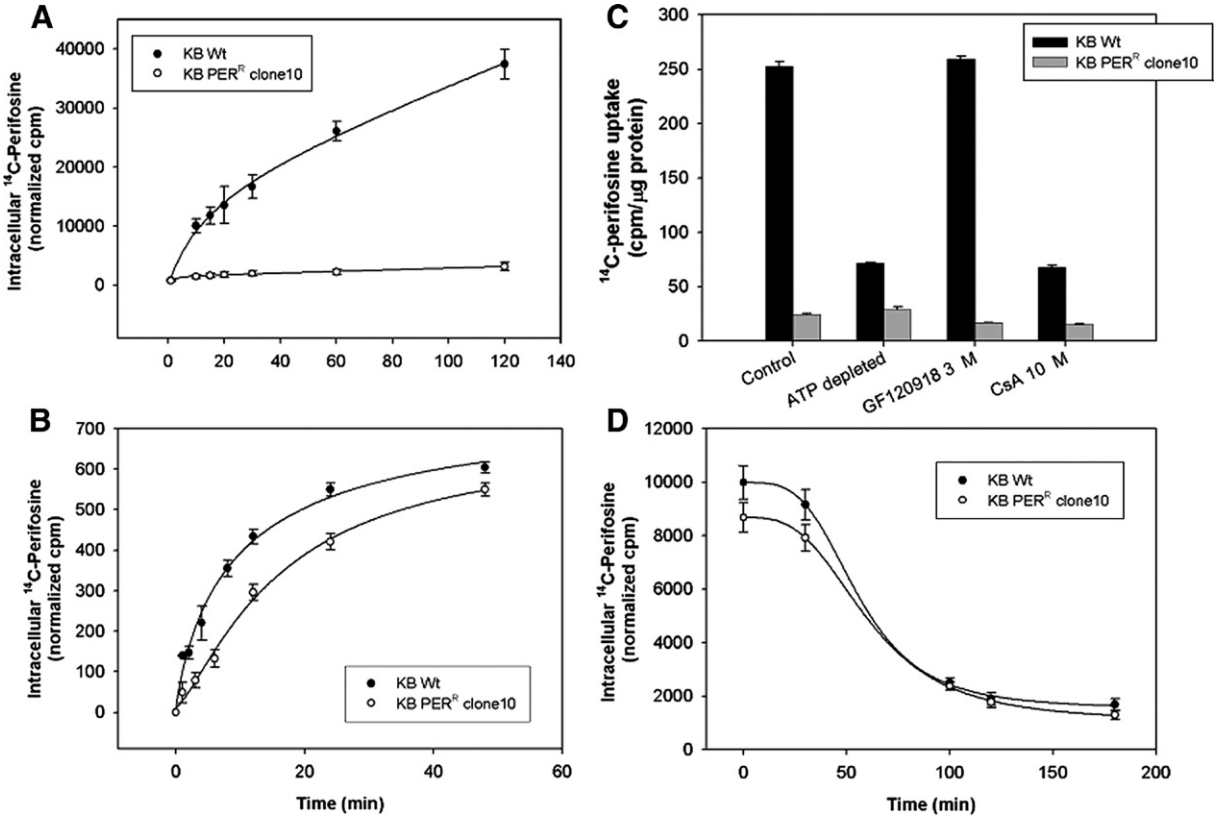

Fig. 2. Uptake of ^{14}C-perifosine (0.03 μCi/ml, 1 μM) in KB Wt and KB PER^R clone10 cells at 37 °C (A,C), at 4 °C (B), and after ATP depletion or in the presence of the ABC transporter inhibitors cyclosporine A (CsA) or GF120918 (C). In (D) is showed the comparison of ^{14}C-perifosine efflux between KB Wt and KB PER^R clone10 cells at 37 °C. Representative experiments of 2–3 different experiments performed in duplicate are showed.

defect of drug internalization was attributable to either endocytosis or membrane translocation, we performed the same experiment but at 4 °C, where no endocytosis occur (Fig. 2B). We observed that ^{14}C-perifosine in Wt cells equilibrated between the two leaflets of the plasma membrane within 30–40 min, with a half-maximal accumulation time ($t_{1/2}$) of around 7 min. However, drug equilibration in the plasma membrane of PER^R clone10 cells took a longer time (around 60 min.), with $t_{1/2} \approx 15$ min. This implies that the perifosine internalization impairment observed in PER^R clone10 cells was, at least in part, due to a lower rate of translocation through the plasma membrane.

Finally, we wanted to check whether the lower levels of perifosine accumulation observed in PER^R clone10 cells were or were not due to an active mechanism of drug extrusion (e.g. over-expression of ABC pumps) absent or present at lower levels in KB Wt cells. We performed experiments of perifosine uptake either after ATP depletion, or in the presence of the ABC transporter inhibitors cyclosporine A and GF120918 (Fig. 2C). Depletion of ATP dramatically inhibited the uptake of perifosine in KB Wt cells at 37 °C, suggesting that such uptake is highly dependent on the cellular ATP levels. On the other hand, uptake of perifosine in KB PER^R was unaffected by ATP depletion, which suggests that no active internalization of perifosine takes place in this cell line (no ATP-dependent translocase). If an active efflux pump for perifosine existed in PER^R cells, the ATP depletion should have raised the uptake of perifosine up to the levels measured in Wt cells in the same experimental conditions; however, this was not observed. The pre-treatment with either cyclosporine A or GF120918, which are inhibitors of three classic efflux pumps that belong to the ABC superfamily of transporters (MDR1/MRP1, and ABCG2/MDR1, respectively), had no effect in the rate of perifosine uptake in KB PER^R cells, which suggest that none of these efflux pumps contribute to the perifosine-resistance phenotype observed in PER^R cells. It is worth noting that cyclosporine A partially inhibited the uptake of perifosine in Wt cells, which could imply that this drug would be able to inhibit the aminophospholipid translocase(s). This observation would explain the reported pro-coagulant effect of cyclosporine A by inducing the exposition of PS in platelets [22].

Both cell lines were loaded with ^{14}C-perifosine, and then allowed to extrude the drug in order to measure possible differences in their respective drug efflux rates. We observed no differences at all in the drug efflux rates between both cell lines (Fig. 2D), which made us to conclude that the lower intracellular accumulation of perifosine in PER^R clone10 with respect to Wt cells was due to a slower rate of drug internalization, rather than an increased drug efflux.

3.3. ALPs compete with the uptake of ^{14}C-perifosine in a concentration-dependent manner

Considering that perifosine seemed to enter in KB cells by translocation through the plasma membrane, we wanted to determine if such translocation process was saturable at increasing concentrations of the drug, which would exclude passive diffusion as well as endocytosis as major mechanisms responsible for perifosine uptake. Instead, saturation would imply the involvement of a putative membrane transporter or receptor. We found that the rate of ^{14}C-perifosine accumulation in KB Wt cells in the presence of 10% FBS (or BSA 0.3%) was linear up to 10–15 μM perifosine, but at higher concentrations the rate started to slow down until complete saturation at 70–80 μM perifosine (not shown). The ^{14}C-perifosine uptake process could be described by a hyperbola with $K_m \approx 18$ μM. Interestingly, when ^{14}C-perifosine was added to the cells in a medium without FBS (FBS-free medium only at the moment of the experiment), we observed that the drug accumulation did not saturate, but was linearly dependent on ^{14}C-perifosine concentration up to at least 160 μM, and it proceed faster than in the presence of 10% FBS (not shown). Strikingly, in the absence of FBS we observed that PER^R clone10 cells showed a similar sensitivity to perifosine than parental cells, and they also took up similar amounts of ^{14}C-perifosine. These findings suggest that perifosine molecules should be complexed with the BSA present in FBS for their proper insertion in the plasma membrane because, in the absence of FBS, perifosine seems to be internalized by a non-saturable process, as previously described for other alkylphospholipids like edelfosine [23].

However, since ALPs can act as detergents and produce membrane fluidification [24] and cell lysis [25] at high concentrations, the observed "saturation" of ^{14}C-perifosine accumulation could be due to non-specific perturbations of the plasma membrane at the higher concentrations of ^{14}C-perifosine used above. Thus, in order to rule out this non-specific effect of ALPs, we performed competition assays of ^{14}C-perifosine in the presence of cold perifosine, miltefosine and edelfosine at

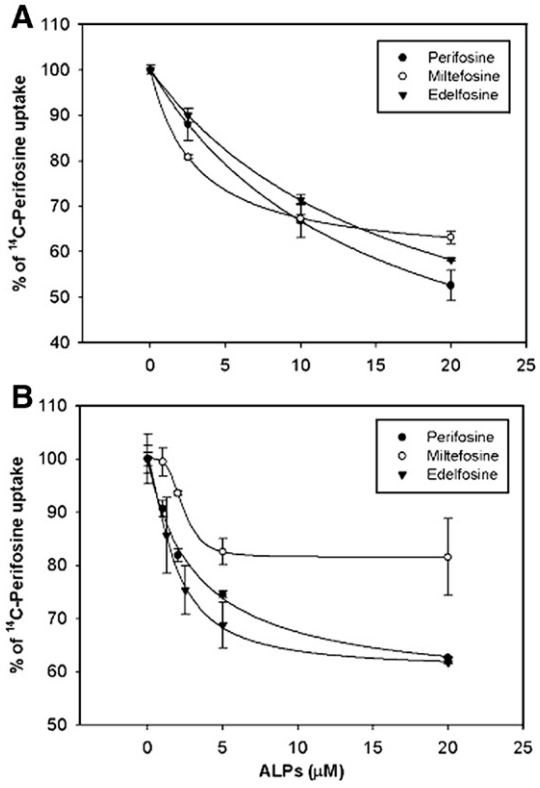

Fig. 3. Inhibition of ^{14}C-perifosine uptake by increasing concentrations of cold perifosine, miltefosine and edelfosine in KB Wt cells at both 4 °C (A) and 37 °C (B).

low concentrations (up to 20 μM). Fig. 3A shows that, at 4 °C, the three ALPs reduced the uptake of ^{14}C-perifosine by 20 to 45% in a concentration-dependent manner. The EC50s (concentration that causes the half-maximal effect observed) were 3.26±0.47 μM, 2.36±0.01 μM and 1.82±0.13 μM for perifosine, miltefosine and edelfosine, respectively. Whatever the mechanism inhibited by ALPs at 4 °C, it was also relevant at 37 °C, as shown in Fig. 3B.

3.4. ^{14}C-perifosine uptake is related to aminophospholipid translocation in human KB cells

Taking into account the structural similarities between ALPs and natural phospholipids at molecular level, and the results exposed above, we considered that P4-type aminophospholipid translocases [26] could be good candidates for putative mediators of ALPs uptake in human KB cells. To test our hypothesis we performed experiments of accumulation of the fluorescent phospholipid analogues NBD-PE, -PS and -PC, monitored by flow cytometry, using both KB Wt and PER^R clone10 cells. The best correlation between perifosine and NBD-phospholipid uptake was found with NBD-PE, rather than with NBD-PS or -PC, as shown in Fig. 4A. We found that PER^R clone10 cells accumulated 10- and 2-fold less NBD-PE than Wt cells at 37 °C (Fig. 4B) and 4 °C (Fig. 4C), respectively, after 20 min incubation, which is in agreement with their respective abilities to translocate perifosine at both 37 °C (Fig. 2A) and 4°C in the linear range (the first 10 min; Fig. 2B) of uptake

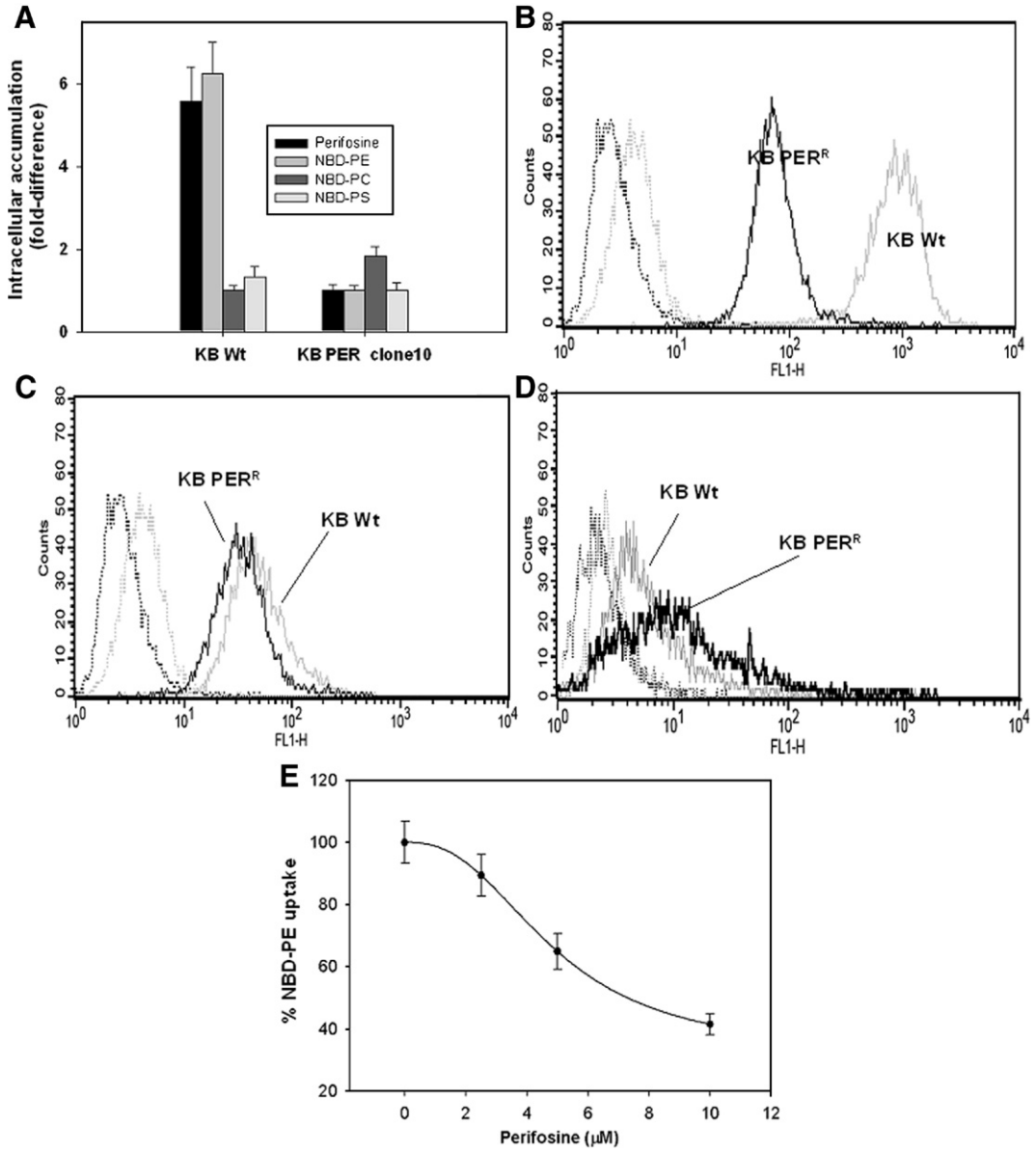

Fig. 4. Accumulation of NBD-phospholipid analogues in KB Wt and KB PER^R clone10 cells: uptake of perifosine compared with that of NBD-PE, -PS and -PC at 37 °C (A); uptake of NBD-PE at 37 °C (B) and 4 °C (C); endogenous phosphatidylserine exposure revealed by Alexa 488-Annexin V binding (D). In (E) is showed the inhibition of NBD-PE uptake at 4 °C by increasing concentrations of perifosine. A representative experiment of two different ones performed in duplicate is shown. In (B), (C) and (D), the respective controls are depicted in dotted lines.

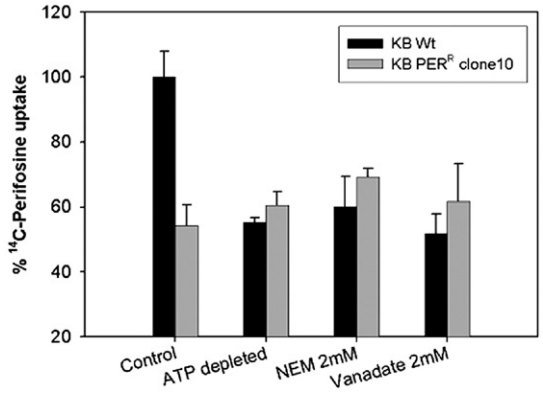

Fig. 5. Uptake of ^{14}C-perifosine at 4 °C in KB Wt is in part dependent on ATP, and sensitive to the P-type aminophospholipid translocases inhibitors NEM and vanadate. Uptake in KB PER^R clone10 cells is lower than in Wt cells, and it is neither an active process (ATP-independent) nor sensitive to NEM and vanadate.

through the plasma membrane. We also observed that Alexa Fluor 488-Annexin V binding to the cell surface was higher in PER^R clone10 cells (Fig. 4D), which implied that these cells exposed more phosphatidylserine than parental KB cells, and that phospholipid asymmetry of these cells should be at least partially disrupted, which suggests a lower aminophospholipid translocase activity in PER^R cells.

Finally, we found that perifosine inhibited in KB Wt cells the internalization of NBD-PE at 4 °C (Fig. 4E) in a concentration-dependent manner up to 10 μM (a concentration low enough as to discard the possibility of membrane-perturbing effects). In a reciprocal way, we found that 10 μM NBD-PE inhibited the uptake of perifosine by about 50%, which, along with the above result, strongly suggest that both perifosine and NBD-PE could be competing for the same transporter to enter KB Wt cells.

3.5. KB Wt displayed an ATP-dependent, NEM- and vanadate-sensitive perifosine-translocase activity absent in PER^R clone10 cells

Given that perifosine uptake in KB cells seemed to be mediated by a membrane aminophospholipid translocase, and that the family of proteins such translocases belong to are NEM- and vanadate-sensitive ATPases [26], we performed ^{14}C-perifosine accumulation experiments at 4 °C (no endocytosis) in both KB Wt and PER^R clone10 cells in ATP-depleted conditions, and in the absence/presence of NEM and orthovanadate. The results of these experiments are depicted in Fig. 5. We found that, after 10 min incubation in control conditions with 1 μM ^{14}C-perifosine, PER^R clone10 cells accumulated only half the amount of perifosine than Wt cells. However, after ATP-depletion or preincubation with either NEM or orthovanadate, the ^{14}C-perifosine uptake in Wt cells decreased to the levels of PER^R clone10 cells. The uptake of ^{14}C-perifosine by PER^R clone10 was unaffected by either ATP-depletion or preincubation with the above-mentioned inhibitors. This demonstrates that in KB Wt cells there is a perifosine-translocase activity driven by ATP, and sensitive to the P-type ATPases inhibitors orthovanadate and NEM. The basal amount of ^{14}C-perifosine accumulated in PER^R clone10 cells at 4 °C seemed to be due to a slower process, driven by the concentration gradient (either passive of protein-facilitated diffusion through the plasma membrane).

3.6. MβCD and chlorpromazine inhibit the uptake of ^{14}C-perifosine in KB Wt cells independently of their role as classical endocytosis inhibitors

Despite that all the evidences gathered to this point of our research claimed for a major role of active membrane translocation in the uptake of ALPs in human KB cells, we also wanted to explore the role of endocytosis in this phenomenon. For such purpose, we performed accumulations of ^{14}C-perifosine after treatment of KB Wt cells with either the cholesterol-depleting agent MβCD (classical inhibitor of raft-dependent endocytosis) or chlorpromazine (classical inhibitor of clathrin-dependent endocytosis).

We found that 10–15 μM chlorpromazine inhibited the ^{14}C-perifosine uptake by 60% at both 37 °C and 4 °C, which is a concentration below the IC50 for inhibition of Alexa Fluor 488-transferrin endocytosis (around 40 μM) (Fig. 6A). Moreover, we tested that chlorpromazine also inhibited translocation of NBD-PE by the same extent at the same concentration range (around 10 μM; not shown). From Fig. 6A it is clear that around 40% of ^{14}C-perifosine uptake remained insensitive to chlorpromazine, but higher concentrations (up to 100 μM) were able

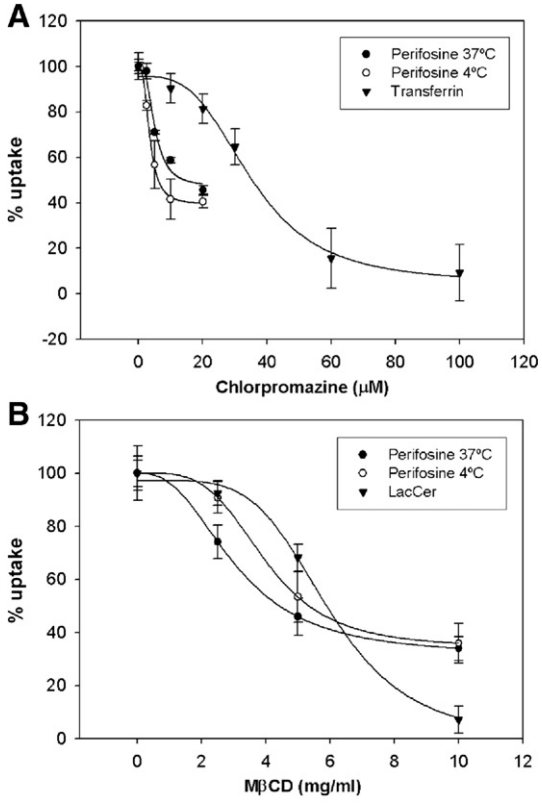

Fig. 6. Effects of chlorpromazine (A) and MβCD (B) in the uptake of ^{14}C-perifosine (at both 37 °C and 4 °C), and in the endocytosis of transferrin and lactosyl-ceramide (LacCer), which are the respective controls of clathrin- and raft-dependent endocytosis.

to completely abolish clathrin-mediated endocytosis. This suggests that clathrin-mediated endocytosis played no role in perifosine uptake in KB cells.

On the other hand, MβCD inhibited completely the raft-dependent endocytosis of BODIPY-lactosylceramide at 10 mg/ml (Fig. 6B). However, around 40% of ^{14}C-perifosine uptake at 37°C remained insensitive to MβCD-induced cholesterol depletion. This could imply that perifosine uptake and raft-dependent endocytosis were not linked in KB cells, or that 60% of the perifosine uptake took place by raft-dependent endocytosis and the remaining 40% was dependent on other mechanism, like active translocation through the plasma membrane. However, perifosine uptake at 4 °C (where no endocytosis occurred) showed the same profile of inhibition by MβCD than at 37 °C. Moreover, we observed also that NBD-PE translocation through the plasma membrane was also sensitive to MβCD, and in the same way as perifosine uptake (half-maximal inhibition at 2–3 mg/ml MβCD, and 40% of uptake remained insensitive to the drug). This suggests that aminophospholipid translocase is partially dependent on the cholesterol-content of the plasma membrane. Moreover, in a different way as described for other cell lines [13,14], raft-dependent endocytosis seems not to play a major role in perifosine uptake in KB cells.

3.7. KB Wt and PER^R clone10 cells displayed similar rates of endocytosis, and knock-down of clathrin and dynamin 1 did not affect perifosine uptake

Raft-dependent endocytosis has been reported to be the mechanism of ALPs uptake in other cell lines [13,14]. However, we found that both Wt and PER^R clone10 cells displayed similar rates of fluid-phase and raft-dependent endocytosis (Fig. 7A). Thus, the lower levels of perifosine uptake in clone10 cells cannot be ascribed to either of both endocytic processes. However, we found that the rate of clathrin-dependent endocytosis was slower in PER^R clone10 cells than in their parental counterparts (Fig. 7A).

Given that, in spite of the above evidences against the role of both clathrin- and raft-mediated endocytosis in perifosine uptake, we wanted to establish definitely the potential contribution of any of these bulk transport processes. We decided to knock down clathrin heavy chain and dynamin 1, both key proteins of the endocytic machinery that play a role in clathrin-dependent [27] and clathrin-independent, raft-dependent routes of endocytosis [28], by using the siRNA-based ON-TARGET-plus approach developed by Dharmacon. We found that, after 48 h of transfection with 100 nM siRNA clathrin mixture, the rates of ^{14}C-perifosine uptake, fluid phase (monitored by Alexa Fluor 488-Dextran 10 KDa uptake) and clathrin-dependent (monitored by Alexa Fluor 488-transferrin uptake) endocytosis, were reduced around 10, 60, and 50%, respectively, versus the control with the cationic lipid mixture alone (Fig. 7B). However, raft-dependent endocytosis (monitored by BODIPY-lactosylceramide uptake) was unaffected by clathrin knock-down (Fig. 7B). On the other hand, after 48 h of transfection with 100 nM siRNA dynamin mixture, the rates of ^{14}C-perifosine uptake, fluid phase, clathrin-dependent and raft-dependent endocytosis were reduced around 10, 60, 40 and 50%, respectively. This result, along with the rest of evidence exposed above, allowed us to reject the role of endocytosis in perifosine uptake in KB cells.

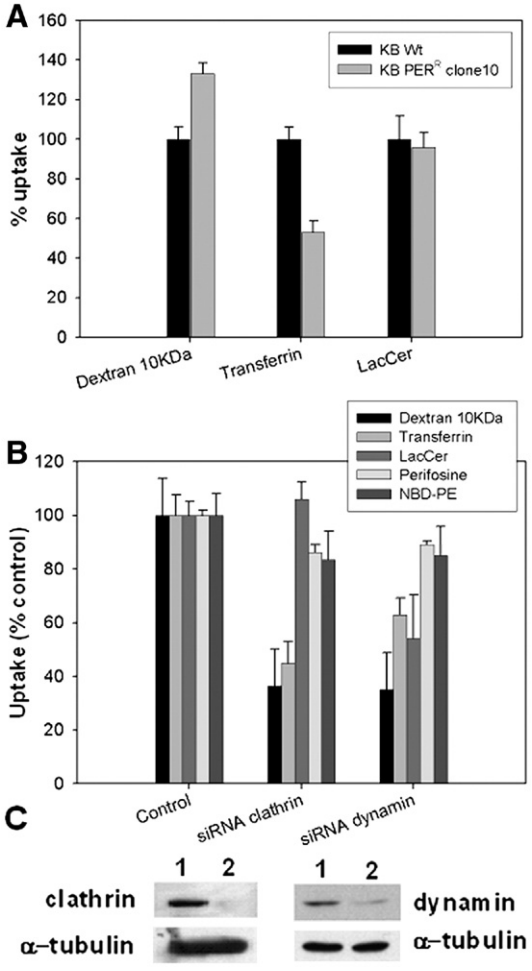

Fig. 7. A) Uptake rates of different endocytic markers compared between KB Wt and KB PER^R clone10 cells. B) Effect of clathrin and dynamin silencing in the uptake of ^{14}C-perifosine, NBD-PE and several endocytic markers. C) Expression levels of clathrin heavy chain and dynamin, determined by Western blot, in control cells (incubated with Dharmafect, lane 1) and siRNA-transfected cells (lane 2).

4. Discussion

In the present work, we have reported the generation of a cell line derived from the perifosine-hypersensitive human KB cells (the KB PER^R clone10 cells), which are highly resistant to the ALP perifosine and cross-resistant to other ALPs like miltefosine and edelfosine, but not to the classical anticancer drug daunorubicin. This cell line showed a marked impairment in perifosine uptake, but no signs for active efflux of the drug or abrogation of apoptosis, since daunorubicin caused rapid cell death in both parental and PER^R clone10 cells with equal potency. Therefore, the impairment in perifosine uptake in PER^R clone10 cells seems to be the major mechanism that accounts for the observed resistance phenotype. We have observed that the differential uptake of perifosine between wild-

type and perifosine-resistant (PER^R) cells requires the presence of either FBS or BSA in the medium. This is not the first time that such observation has been reported: Small et al. [23] found that edelfosine-resistant HL-60 cells accumulated similar amounts of edelfosine than sensitive cells in the absence of serum albumin. It has been demonstrated that alkylphospholipids (ALPs), below the critical micellar concentration (CMC), insert progressively in lipid monolayers as monomers from the aqueous medium, but above the CMC, an irregular insertion of ALP molecules occurs, because not only monomers but also groups of monomers are transferred into the monolayers [29]. It is known that the presence of albumin in the medium has the effect of increasing the CMC value by binding molecules of lipids and, hence, reducing the concentration of free monomers in the medium [30, 31]. Albumin acts as a perifosine reservoir [31]; it binds reversibly to the cell surface and may release the drug gradually. Therefore, the presence/absence of BSA clearly determines the way in which perifosine interacts with the plasma membrane of living cells. With BSA, perifosine will insert in the outer leaflet of the plasma membrane as monomers, but will pass through the membrane bilayer only where the putative ATP-dependent translocase is active (in the membrane of wild-type cells, but not in that of PER^R cells). However, without BSA, abrupt deployment of perifosine micelles into the plasma membrane will occur as soon as the CMC is reached, overcoming in PER^R cells the barrier imposed by the absence of a translocase for perifosine, which make them as sensitive to the drug as KB Wt cells. This could also explain the observed low levels of competition between ^{14}C-perifosine and perifosine, miltefosine and edelfosine (never greater than around 50%), since effective competition could only take place when the molecules of the competitor ALP are as free monomers; once the CMC is reached, the ALP molecules tend to cluster in micelles and a real competition between hot and cold molecules of ALPs cannot proceed further.

We investigated whether perifosine entered KB cells by endocytosis, as previously reported for other cell lines [13,14], and found that both chlorpromazine (classical inhibitor of clathrin-dependent endocytosis) and MβCD (classical inhibitor of raft-dependent endocytosis) reduced the uptake of perifosine considerably. In principle, a simple interpretation of these results would make us conclude that both clathrin- and raft-dependent endocytosis played a major role in perifosine uptake in KB cells. However, we wanted to make certain that the above-mentioned inhibitors were not causing effects other than the inhibition of endocytosis. We observed that the membrane translocation of both NBD-PE and perifosine at 4 °C was inhibited by chlorpromazine at concentrations below the EC50 for inhibition of clathrin-dependent endocytosis, as monitored with Alexa Fluor 488-transferrin uptake. Moreover, we found that: first, MβCD inhibited the translocation of both NBD-PE and perifosine at 4 °C; second, that perifosine uptake at both 4 °C and 37 °C showed a similar profile of sensitivity to MβCD; and third, that 40% of the perifosine uptake remained insensitive to MβCD even at the highest concentrations used (10 mg/ml), but the uptake of BODIPY-lactosylceramide (marker of raft-dependent endocytosis) was inhibited completely. We wanted to explore the effects of higher concentrations of MβCD (5–10 mg/ml) than those used in previous works [13,14], because we observed that MβCD at 1 to 2.5 mg/ml was unable to affect endocytosis of BODIPY-LacCer in KB cells (Fig. 6B), which suggested that membrane rafts were still unaffected and that higher concentrations of MβCD were necessary to cause raft-disruption without affecting cell integrity. Some authors could criticise that the high concentrations of MβCD used (5–10 mg/ml) could be toxic for cells. We do not doubt that raft disruption should be deleterious for living cells, but the toxic effects should not be apparent until several hours after MβCD treatment. Indeed, we accumulated ^{14}C-perifosine immediately after MβCD treatment for no longer than 1 h, and the cells examined under the microscope looked perfectly well before and after such interval. Moreover, 40% of the active accumulation of ^{14}C-perifosine remained insensitive to MβCD pre-treatment, which means that the cell membrane as a physical barrier should be intact even at the highest concentration of MβCD used. Otherwise, the active accumulation of the drug could not proceed. Finally, it is worth noting that ^{14}C-perifosine uptake at 37 °C is significantly lowered after pre-treatment with only 2.5 mg/ml MβCD, but endocytosis of BODIPY-LacCer was still unaffected.

All these findings suggested that chlorpromazine and MβCD were able not only to inhibit endocytosis, but also to block perifosine and aminophospholipid translocation through the plasma membrane in a concentration range that did not affect endocytosis. In addition, it seemed that perifosine uptake in KB cells was occurring by a process other than endocytosis, which was confirmed when we silenced dynamin and found no effect in the uptake of perifosine. Therefore, endocytosis is not responsible for perifosine uptake in KB cells.

KB PER^R clone10 cells showed no major alterations in endocytosis with respect to parental KB cells, which supports what was stated above. Only the endocytosis of Alexa Fluor 488-transferrin (clathrin-dependent) seemed to be lower in PER^R clone10 cells but, since dynamin plays a key role in clathrin-dependent endocytosis and its silencing caused no impairment in perifosine uptake in KB cells, we could conclude that the lower levels of clathrin-dependent endocytosis observed in PER^R clone10 cells cannot explain the lower perifosine uptake. Moreover, we found that perifosine uptake was saturable, and that perifosine, miltefosine, and edelfosine inhibited the uptake of ^{14}C-perifosine in a concentration-dependent manner. This suggested that perifosine uptake should be mediated by a discrete machinery (either a transporter or a receptor), and that whatever is the protein or complex of proteins affected in PER^R clone10 cells, it should be responsible for the uptake of all ALPs in KB cells.

Taken into account the similarities between ALPs and natural phospholipids, we considered it plausible that whatever is the putative transporter implicated in the uptake of ALPs in KB cells, it could be affected in KB PER^R clone10 cells. We found that PER^R clone10 cells present a reduced uptake of NBD-PE and an increased exposure of phosphatidylserine in the surface of the plasma membrane with respect to the parental cells. Given that it is long since known that uptake of spin-labeled

[32], unlabelled short chain [33], and fluorescent aminophospholipid analogues [34,35] in mammalian cells is mediated by an ATP-dependent membrane translocase, these findings suggest that an aminophospholipid translocase activity, which contributes to create the phospholipid asymmetric distribution typical in the plasma membrane of any eukaryotic cells [26,36] was absent or greatly lowered in PER^R clone10 cells. Unsurprisingly, we were able to establish that perifosine translocation at 4 °C in KB cells was an active process, dependent on ATP and sensitive to NEM and orthovanadate, which are the hallmarks of the P-type ATPases like aminophospholipid translocases [26]. Even more interestingly, such activity was absent in PER^R clone10 cells, and perifosine inhibited the translocation of NBD-PE in a concentration-dependent manner, which suggests that both natural aminophospholipids and ALPs compete for the same transporter to be internalized in KB cells.

In conclusion, the results presented in this work point towards the implication of a putative aminophospholipid translocase in the uptake of ALPs in human KB cells, as it has also been described previously in yeasts [37] and protozoan parasites [38]. Moreover, the present work points out, as previous works also did [39,40], that great care should be taken when using chlorpromazine and MβCD as endocytosis inhibitors, since these compounds seem to have pleiotropic effects (like the inhibition of aminophospholipid translocases observed here), and could lead researchers to link erroneously a given phenomenon to endocytosis.

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