A new class of anticancer alkylphospholipids uses lipid rafts as membrane gateways to induce apoptosis in lymphoma cells

Arnold H. van der Luit,[1] Stefan R. Vink,[2] Jeffrey B. Klarenbeek,[1] Daniel Perrissoud,[4] Eric Solary,[5] Marcel Verheij,[1,3] and Wim J. van Blitterswijk[1]

Divisions of [1]Cellular Biochemistry and [2]Experimental Therapy, [3]Department of Radiotherapy, the Netherlands Cancer Institute, Plesmanlaan 121, Amsterdam, the Netherlands; [4]Zentaris GmbH, Frankfurt am Main, Germany; and [5]Institut National de la Sante et de la Recherche Medicale U866/Université de Bourgogne, Laboratoire de Mort Cellulaire et Cancer, UFR de Médecine, Dijon, France

Abstract

Single-chain alkylphospholipids, unlike conventional chemotherapeutic drugs, act on cell membranes to induce apoptosis in tumor cells. We tested four different alkylphospholipids, i.e., edelfosine, perifosine, erucylphosphocholine, and compound D-21805, as inducers of apoptosis in the mouse lymphoma cell line S49. We compared their mechanism of cellular entry and their potency to induce apoptosis through inhibition of *de novo* biosynthesis of phosphatidylcholine at the endoplasmic reticulum. Alkylphospholipid potency closely correlated with the degree of phosphatidylcholine synthesis inhibition in the order edelfosine > D-21805 > erucylphosphocholine > perifosine. In all cases, exogenous lysophosphatidylcholine, an alternative source for cellular phosphatidylcholine production, could partly rescue cells from alkylphospholipid-induced apoptosis, suggesting that phosphatidylcholine biosynthesis is a direct target for apoptosis induction. Cellular uptake of each alkylphospholipid was dependent on lipid rafts because pretreatment of cells with the raft-disrupting agents, methyl-β-cyclodextrin, filipin, or bacterial sphingomyelinase, reduced alkylphospholipid uptake and/or apoptosis induction and alleviated the inhibition of phosphatidylcholine synthesis. Uptake of all alkylphospholipids was inhibited by small interfering RNA (siRNA)–mediated blockage of sphingomyelin synthase (SMS1), which was previously shown to block raft-dependent endocytosis. Similar to edelfosine, perifosine accumulated in (isolated) lipid rafts independent on raft sphingomyelin content per se. However, perifosine was more susceptible than edelfosine to back-extraction by fatty acid-free serum albumin, suggesting a more peripheral location in the cell due to less effective internalization. Overall, our results suggest that lipid rafts are critical membrane portals for cellular entry of alkylphospholipids depending on SMS1 activity and, therefore, are potential targets for alkylphospholipid anticancer therapy. [Mol Cancer Ther 2007;6(8):2337–45]

Introduction

Synthetic lipase-resistant analogues of lysophosphatidylcholine, collectively named alkylphospholipids, exert cytotoxic effects against a wide variety of tumors (1–4). The prototype of these compounds, 1-*O*-octadecyl-2-*O*-methyl-*rac*-glycero-3-phosphocholine (Et-18-OCH_3, edelfosine), structurally resembles lysophosphatidylcholine (Fig. 1) in that it has the same polar head group and a single, long apolar hydrocarbon chain, which allows an easy insertion into the plasma membrane. Whereas membrane-inserted lysophosphatidylcholine undergoes rapid turnover, edelfosine with its stable ether bonds is not metabolized, leading to accumulation in cell membranes. This interferes with lipid-based signal transduction, often resulting in apoptosis of tumor cells. In later studies, the glycerol moiety in alkylphospholipid was found not essential for the antitumor activity. A second generation of these compounds, therefore, comprised of phospho-ester–linked (single-chain) alkylphosphocholines and derivatives thereof. The first of these compounds, hexadecylphosphocholine (miltefosine), was clinically effective in patients with skin metastasis of breast cancers (2, 5) and cutaneous lymphomas (6). Erucylphosphocholine containing a much longer (22-carbon) chain with a cis-13,14 double bond (Fig. 1) could be applied i.v. and thereby showed improved antitumor activity with reduced hemolytic and gastrointestinal side effects (7, 8). Interestingly, it increased the cytotoxicity of ionizing radiation (9, 10). A structural analogue, octadecyl-(1,1-dimethyl-4-piperidynio-4-yl)-phosphate (perifosine; D-21266), in which the choline head group has been substituted by a piperidine moiety (Fig. 1) has received mounting attention as an anticancer agent, especially in combination with other pharmacologic drugs (11–16) as well as with radiotherapy (4, 17–19).

Received 3/22/07; revised 5/16/07; accepted 6/12/07.

Grant support: Dutch Cancer Society grants NKI 2001-2570 and NKI 2005-3377.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Note: Current address for A. H. van der Luit: Oncodesign, 20 rue Jean Mazen, B.P. 27627, 21076 Dijon Cedex, France.

Requests for reprints: Wim J. van Blitterswijk, Division of Cellular Biochemistry, the Netherlands Cancer Institute, Plesmanlaan 121, 1066 CX Amsterdam, the Netherlands. Phone: 31-205121976; Fax: 31-205121989. E-mail w.v.blitterswijk@nki.nl

doi:10.1158/1535-7163.MCT-07-0202

In contrast to classic chemotherapeutic drugs that target the DNA, alkylphospholipids act at cell membranes by interfering with the turnover and synthesis of natural phospholipids and by disrupting membrane-signaling networks at multiple sites, leading to cell death (3, 4, 20). For the alkylphospholipids edelfosine and miltefosine, it has been shown that they induce apoptosis through the inhibition of CTP:phosphocholine cytidylyltransferase (CT), a key enzyme in phosphatidylcholine biosynthesis (21–24). To inhibit this enzyme at the endoplasmic reticulum, alkylphospholipids need to be internalized. We have shown that edelfosine, after insertion in the plasma membrane, accumulates in lipid rafts and is then internalized by an endocytotic pathway that depends on intact rafts and on the activity of sphingomyelin synthase 1 (23–25). How other alkylphospholipids are taken up by cells, their relative potency and mechanism of apoptosis induction is currently unknown.

Here, we compared four anticancer alkylphospholipids (molecular structures depicted in Fig. 1) with respect to their cellular uptake, cytotoxicity, and mechanism of apoptosis induction in mouse S49 lymphoma cells. We found that all alkylphospholipids use lipid rafts for internalization to inhibit phosphatidylcholine synthesis to varying degrees, correlating with the efficiency and persistency of cellular uptake and potency to induce apoptosis.

Materials and Methods

Reagents

Edelfosine (1-*O*-octadecyl-2-*O*-methyl-*rac*-glycero-3-phosphocholine; Et-18-OCH_3) was purchased from BioMol. [^{3}H]Edelfosine ([^{3}H]Et-18-OCH_3; 40 Ci/mmol), was synthesized by Moravek Biochemicals. The other alkylphospholipids (Fig. 1), perifosine (compound D-21266), octadecyl-2-(trimethylarseno)-ethyl-phosphate (compound D-21805), erucylphosphocholine, and [^{14}C]perifosine (30.9 mCi/mmol), were kindly provided by Zentaris GmbH. [Methyl-^{14}C]choline chloride (58 mCi/mmol) was from Amersham Pharmacia Biotech. [^{3}H]sphingosine was synthesized by Piet Weber at DSM. Reagents for lipid extraction and subsequent analyses, as well as silica 60 TLC plates (20 × 20 cm), were from Merck. Sphingomyelinase (*Bacillus cereus*), filipin, and methyl-β-cyclodextrin (MβCD) were from Sigma Chemicals Co.

Cell Culture

Mouse S49.1 lymphoma cells (S49) were grown in DMEM, containing high glucose and pyruvate, supplemented with 8% FCS, 2 mmol/L L-glutamine, 100 units/mL penicillin, and 100 μg/mL streptomycin at 37°C and 5% CO_2. Edelfosine-resistant variants (S49^{AR}) were isolated in two selection rounds of growth in 15 μmol/L edelfosine for 72 h, followed by plating in semisolid medium and isolation of colonies of surviving cells (26). S49^{AR} cells could be grown continuously in 15 μmol/L edelfosine with a doubling time of 12 h, similar to that of the parent S49 cells. All experiments with S49^{AR} cells were done with cells grown without the selection agent for at least 1 week.

Silencing of Sphingomyelin Synthase-1 by Small Interfering RNA Retroviral Transduction

To suppress the expression of sphingomyelin synthase-1 (SMS1), S49 cells were retrovirally transduced by small interfering RNA (siRNA), yielding S49^{siSMS1} cells, as described previously (25). Briefly, siRNAs directed to SMS1 were inserted into the retroviral vector pRETRO-SUPER. Retroviral supernatants were obtained from Phoenix cells and used to transduce S49 cells. Stable S49^{siSMS1} cells were selected with puromycin. The following siRNA primers were used: sense GATCCCCGCATGGGAGTTGATTTAGATTCAAGAGATCTAAATCAACTCCCATGCTTTTTGGAAA and antisense AGCTTTTCCAAAAAGCATGGGAGTTGATTTAGATCTCTTGAATCTAAATCAACTCCCATGCGGG. For mock transfection (S49^{mock}), scrambled RNA was used.

Cellular Uptake of Alkylphospholipids and Apoptosis Assay

Cells were grown to a density of 2.5 × 10^6/mL and ^{14}C-perifosine (0.2 μCi, 20 μmol/L) or ^{3}H-edelfosine (0.2 μCi, 15 μmol/L) was added. At given time points, samples were taken, incubated for 2 min on ice, and washed with cold PBS. Samples were lysed in 0.1 N NaOH for scintillation counting. For apoptosis, cells were seeded at 1.5 × 10^6 cells/mL, cultured overnight, and incubated for indicated time periods with various concentrations of alkylphospholipids. Cells were washed with PBS in and lysed overnight at 4°C in 0.1% (w/v) sodium citrate, 0.1% (v/v) Triton X-100, and 50 μg/mL propidium iodide (27). Fluorescence intensity of propidium iodide–stained DNA was determined on a FACScan (Becton Dickinson Advanced Cellular Biology), and data analyzed using Lysis software.

Lipid Biosynthesis

To measure phosphatidylcholine and sphingomyelin biosynthesis, cells at 2.5 × 10^6 cells/mL were incubated with [^{14}C]choline chloride (1 μCi/mL). At given time points, aliquots of cell suspension were taken, washed, and resuspended in 200 μL PBS. Lipids were extracted with chloroform/methanol (1:2, v/v), and phase separation was induced using 1 mol/L 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). Alternatively, cells were radiolabeled for 24 h with 1 μCi/mL [^{3}H]sphingosine (28). In this case, lipid extracts were separated by TLC using chloroform/methanol/0.2% $CaCl_2$ (60:40:9, v/v/v). Radioactive lipids were visualized and quantified using a Fuji BAS 2000 TR Phosphor-Imager and identified using internal standards, which were visualized by iodine staining.

Isolation of Lipid Rafts

A detergent-resistant lipid raft fraction was prepared as described (23). Briefly, cells (2.0 × 10^6/mL) were solubilized into 2 mL of ice-cold MBS buffer (25 mmol/L MES, 150 mmol/L NaCl), including 1% Triton X-100, and homogenized with a tight-fitting Dounce homogenizer (10 strokes). The homogenate was adjusted to 40% sucrose and put on the bottom of an ultracentrifuge tube (4 mL). A discontinuous sucrose gradient was prepared by overlaying 5 mL of 30% sucrose and 3 mL of 5% sucrose (both in MBS), subsequently.

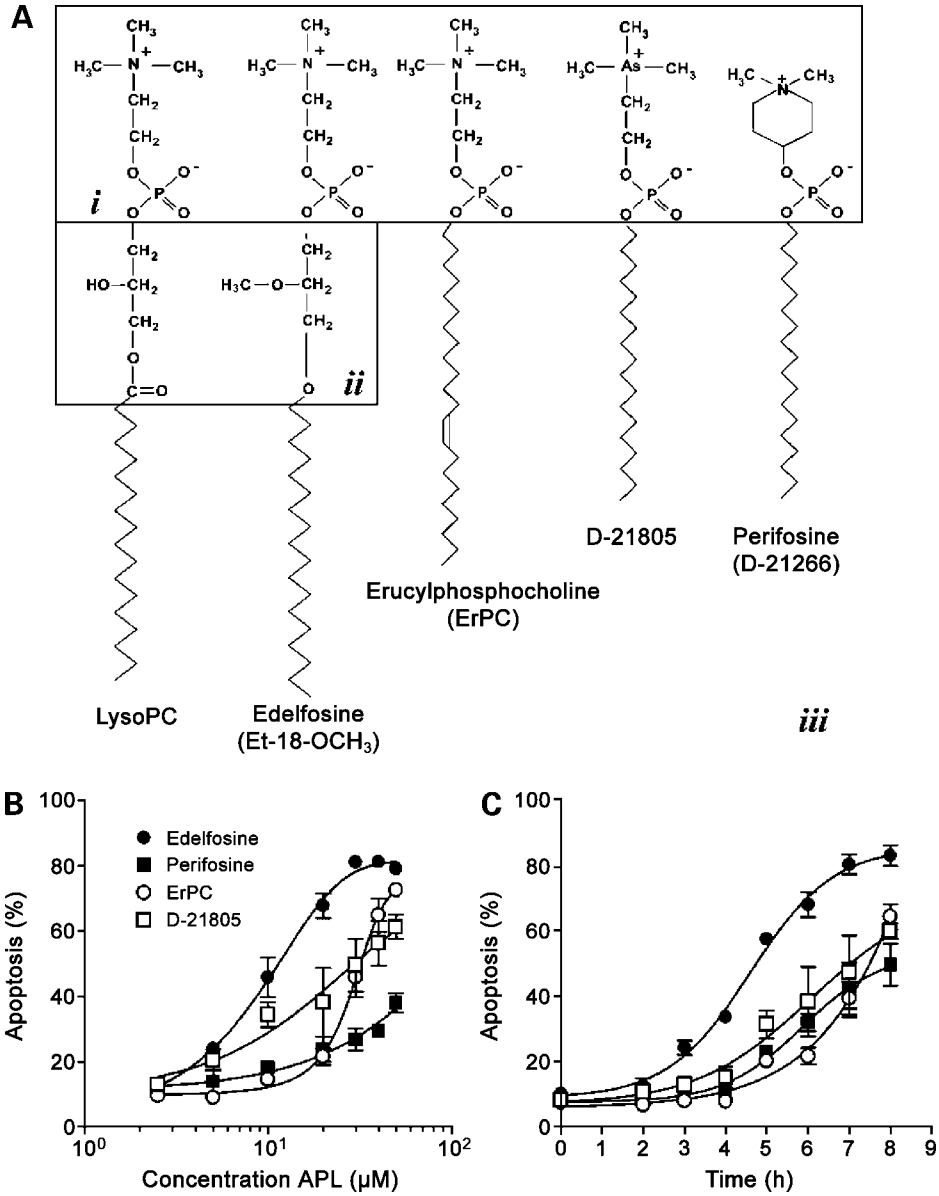

Figure 1. Apoptosis induction in S49 lymphoma cells by various alkylphospholipids. **A,** chemical structures of synthetic alkylphospholipids used in this study. Structures of edelfosine (also denoted as Et-18-OCH_3), erucylphosphocholine, octadecyl-2-(trimethylarsonio)-ethylphosphate (D-21805), and perifosine (D-21266) in comparison to natural lysophosphatidylcholine (*LysoPC*). Note the three distinct submolecular structural moieties, (*i*) phosphocholine and related head group analogs, which are zwitterionic and represent the polar part of the molecule; (*ii*) glycerol backbone, present only in lysophosphatidylcholine and edelfosine; and (*iii*) the apolar alkyl chain (acyl chain in lysophosphatidylcholine). **B,** dose dependency of apoptosis induction by these alkylphospholipids. S49 cells were treated for 6 h with different concentrations of the alkylphospholipids (APL). **C,** Time dependency of apoptosis induction in S49 cells by D-21805 (20 μmol/L; □), perifosine (20 μmol/L, ■), erucylphosphocholine (20 μmol/L; ○), or edelfosine (15 μmol/L Et-18-OCH_3; ●). Apoptotic nuclear fragmentation was determined by FACScan analysis (see Materials and Methods). Data are means of four experiments ± SD.

The tubes were centrifuged at 39,000 rpm in a SW41 rotor for 16 to 18 h at 4°C and 12 × 1.0-mL fractions were collected manually from the top of the gradient. For incorporation of perifosine in lipid rafts, cells were incubated with [^{14}C]perifosine (0.2 μCi/mL; 20 μmol/L) for 15 min to allow insertion into the outer leaflet of the plasma membrane lipid bilayer. Sphingomyelin levels in each fraction were determined after 24 h radiolabeling of cells with 1 μCi/mL [^{3}H]sphingosine, followed by Triton X-100 solubilization and sucrose gradient centrifugation and TLC separation (see above).

Results

Differential Potency of Four Different Alkylphospholipids to Induce Apoptosis in S49 Cells

We have previously described that the synthetic etherlipid edelfosine (Et-18-OCH_3) can induce apoptosis in S49 lymphoma cells in a dose- and time-dependent fashion (23). The onset of apoptosis in these cells was relatively fast and already apparent after 3 h. Because the structurally related analogues perifosine and erucylphosphocholine (Fig. 1A) have more clinical potential, we examined the potency of these compounds, compared with edelfosine, to induce apoptosis induction in S49 lymphoma cells. We also tested a relatively new alkylphospholipid derivative, Zentaris compound D-21805, in which the nitrogen in the choline moiety is substituted by arsenic (Fig. 1A). It seemed that D-21805, erucylphosphocholine and perifosine are less potent inducers of apoptosis than edelfosine, with IC_{50} values of 15, 25, ≥50, and 12 μmol/L, respectively (Fig. 1B). Contrary to edelfosine, D-21805 and perifosine did not reach plateau levels of apoptosis after 7 h incubation, even at a high

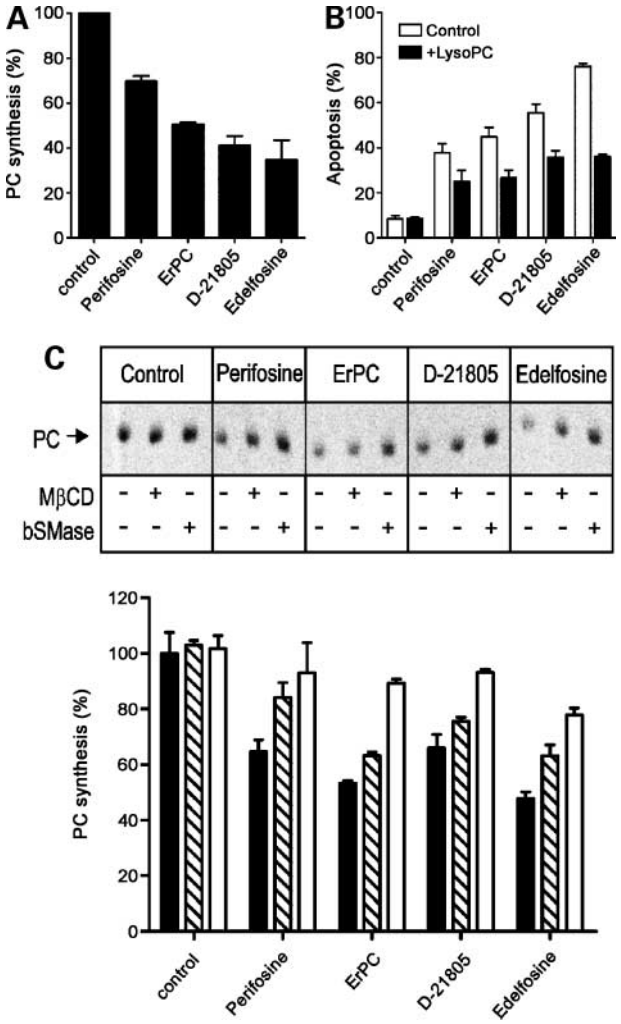

Figure 2. Differential raft-dependent inhibition of phosphatidylcholine synthesis by the various alkylphospholipids correlating with their potency to induce apoptosis. **A,** S49 cells were pretreated for 30 min with perifosine (20 μmol/L), erucylphosphocholine (*ErPC*, 20 μmol/L), D-21805 (20 μmol/L), or edelfosine (15 μmol/L) and then incubated for 2 h with 0.2 μCi/mL [^{14}C]choline precursor to label cellular phosphatidylcholine, which was visualized after lipid extraction and TLC analysis and was quantified using phosphor-imaging technology. **B,** apoptosis induction by alkylphospholipids suppressed by exogenous lysophosphatidylcholine. Apoptosis (nuclear fragmentation) is induced in S49 cells by the various alkylphospholipids (at concentrations given above) after 5 h (*open columns*), and upon coaddition of 25 μmol/L lysophosphatidylcholine (*closed columns*). **C,** alkylphospholipid-induced inhibition of phosphatidylcholine synthesis is alleviated by disruption of lipid rafts. S49 cells were preincubated (for 30 min) with MβCD (2 mg/mL; *hatched columns*) or with bSMase (150 milliunits/mL; *open columns*) or were not preincubated (*black columns*). Cells were then treated with buffer (*control*) or with alkylphospholipids (at concentrations given above) and were, 30 min later, incubated for 2 h with 0.2 μCi/mL [^{14}C]choline precursor to label cellular phosphatidylcholine, which was visualized after lipid extraction and TLC analysis; radioactive phosphatidylcholine spots (*top*) were quantified using PhosphorImaging (*bottom*).

dose of 50 μmol/L (Fig. 1B). In addition, the onset of apoptosis in cells treated with D-21805, erucylphosphocholine, and perifosine was delayed compared with edelfosine (Fig. 1C). Whereas maximal apoptosis by edelfosine was reached at 7 h, the other alkylphospholipids required a prolonged (overnight) incubation time to reach maximum apoptosis.

Relative Potency of Individual Alkylphospholipids to Inhibit Phosphatidylcholine Synthesis Correlates with Their Potency to Induce Apoptosis

We have previously shown, in S49 and HeLa cells, that continuous phosphatidylcholine synthesis is crucial for cell survival, and that edelfosine induces apoptosis by inhibiting *de novo* phosphatidylcholine synthesis (23, 24). To test whether this holds true for the three other alkylphospholipids, S49 cells were incubated with the phosphatidylcholine precursor [^{14}C]choline in the absence or presence of edelfosine, D-21805, perifosine, or erucylphosphocholine, and the phosphatidylcholine synthesized was measured

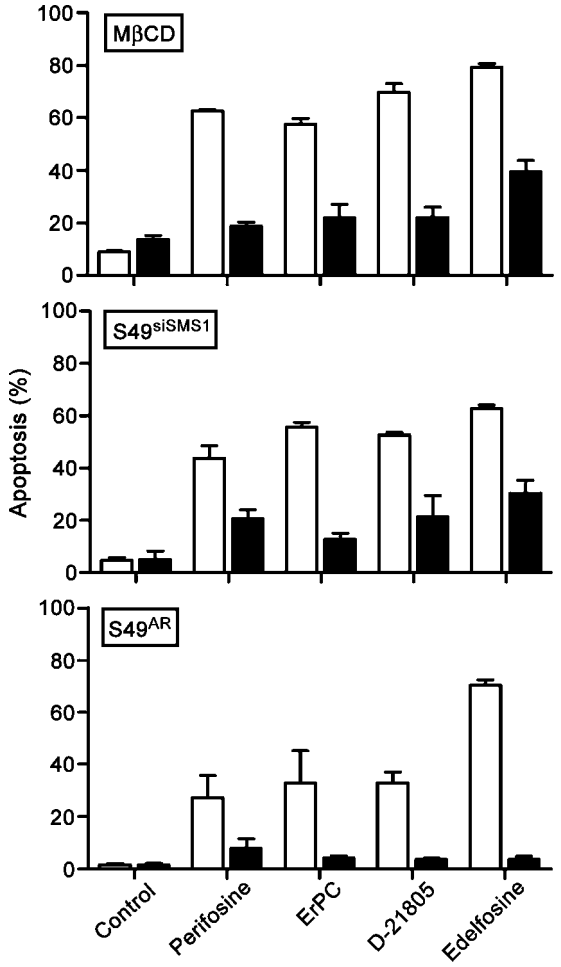

Figure 3. Apoptosis induction by alkylphospholipids is prevented by cholesterol extraction and down-regulation of sphingomyelin synthesis. Apoptosis induced at 7 h by the indicated alkylphospholipids in S49 cells (*open columns*), in S49 cells preincubated (30 min) with MβCD (2 mg/mL; *top, solid columns*) and in the SMS1-deficient cell lines S49^{siSMS1} (*middle, solid columns*) and S49^{AR} (*bottom solid columns*). Perifosine, erucylphosphocholine (*ErPC*), and D-21805 were used at 20 μmol/L; edelfosine was used at 15 μmol/L. Apoptosis was measured by nuclear fragmentation (FACScan analysis; see Materials and Methods). Data are means of triplicates ± SD.

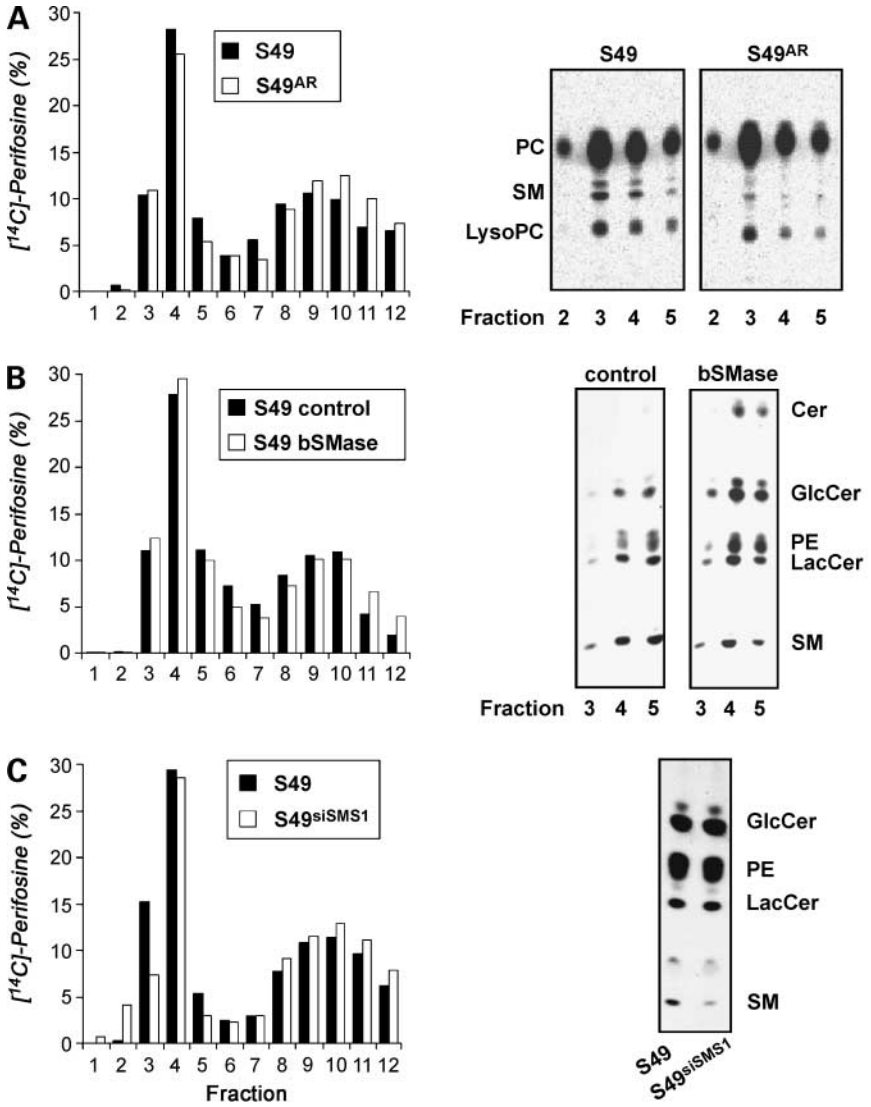

Figure 4. Perifosine accumulates in lipid rafts independent of the sphingomyelin content. *Left,* S49 control cells (*solid columns*) or sphingomyelin-deficient cells ($S49^{siSMS1}$, $S49^{AR}$, and bSMase-treated S49 cells; *open columns*) were incubated for 15 min with [^{14}C]perifosine (20 μmol/L, 0.02 μCi/mL) and washed, and detergent-insoluble lipid rafts were then isolated on sucrose density gradients (typically fractions 3–5). Radioactivity in the gradient fractions was counted and expressed as a percentage of total. **A,** *right,* S49 and $S49^{AR}$ cells (indicated) were labeled with [methyl-^{14}C]choline (1 μCi/mL, overnight). Detergent-insoluble lipid rafts were isolated on sucrose gradients, extracted, and separated by TLC. Positions of phospholipids are indicated. Sphingomyelin is typically represented by two spots (25). **B** and **C,** *right,* S49 cells or $S49^{siSMS1}$ cells were labeled with [^{3}H]sphingosine (1 μCi/mL, 4 h). S49 cells were then treated with bacterial sphingomyelinase (bSMase, 150 milliunits/mL, 30 min; **B**). (Sphingo)lipids were extracted and separated by TLC. The location of sphingomyelin and other sphingolipids, ceramide (*Cer*), glucosylceramide (*GlcCer*), and lactosylceramide (*LacCer*) is indicated. Phosphatidylethanolamine (*PE*) is a catabolic end-product of sphingosine-1-phosphate degradation (25).

after 2 h. The alkylphospholipids were found to inhibit phosphatidylcholine synthesis to various degrees, edelfosine being the most potent, followed by D-21805, erucylphosphocholine, and perifosine, amounting to 65%, 60%, 50%, and 30% inhibition, respectively (Fig. 2A). These data correlate with the relative percentages of apoptosis induced by these compounds (Figs. 1B and 2B).

To show the importance of phosphatidylcholine synthesis for cell survival, we administered exogenous lysophosphatidylcholine, an alternative precursor of phosphatidylcholine (23), to the cells that were challenged with the various alkylphospholipids. Figure 2B shows that, indeed, lysophosphatidylcholine rescued cells from apoptosis induction by perifosine, D-21805, erucylphosphocholine, and edelfosine by 34%, 36%, 40%, and 50%, respectively.

From these results together, we conclude that all four alkylphospholipids inhibit *de novo* phosphatidylcholine synthesis in S49 cells to different degrees, which seemed to correlate with their potency to induce apoptosis. The observed protective effect of lysophosphatidylcholine indicates that this abrogation of phosphatidylcholine synthesis is responsible (at least partly) for the onset of apoptosis induction by all tested alkylphospholipids, like what we concluded previously for edelfosine (23, 24).

Cellular Uptake of Alkylphospholipids, Phosphatidylcholine Synthesis Inhibition, and Consequent Apoptosis Induction Is Mediated by Lipid Rafts

Inhibition of *de novo* phosphatidylcholine synthesis by alkylphospholipids occurs at the level of the rate-determining enzyme CTP:phosphocholine cytidylyltransferase located in the endoplasmic reticulum and the nucleus. Thus, alkylphospholipids need to be internalized to inhibit this enzyme. Edelfosine was previously found to accumulate preferentially in detergent-resistant lipid raft fractions and was internalized by raft-dependent endocytosis (23–25).

To investigate whether phosphatidylcholine inhibition and apoptosis induction in S49 cells by perifosine, D-21805, and erucylphosphocholine was likewise mediated by lipid rafts, we disrupted these membrane domains by extracting their cholesterol with MβCD and by hydrolyzing their sphingomyelin with bacterial sphingomyelinase (bSMase). Figure 2C shows that these treatments of cells alleviated the inhibition of phosphatidylcholine synthesis for each alkylphospholipid. In general, the phosphatidylcholine synthesis–rescuing effect of bSMase treatment was stronger than of MβCD. Figure 3 (*top*) shows that treatment of S49 cells with MβCD prevented apoptosis induction for all tested alkylphospholipids.

We recently described that edelfosine-resistant cells ($S49^{AR}$ cells) lack the raft constituent sphingomyelin through down-regulated sphingomyelin synthase (SMS1) expression. Genetically, sphingomyelin deficiency can also be induced in S49 cells by RNAi-mediated SMS1 down-regulation (Fig. 4C, *right*), yielding so-called $S49^{siSMS1}$ cells (25). Figure 3 (*middle* and *bottom*) shows that both of these S49 cell variants are not only resistant to edelfosine, but to the other alkylphospholipids as well. Resistance in $S49^{siSMS1}$ cells was less stringent than in the $S49^{AR}$ cells because the RNAi-induced down-regulation of SMS1 expression (6-fold reduced mRNA) was not as pronounced as in $S49^{AR}$ cells (50-fold reduced mRNA; ref. 25). The data presented in Fig. 3 confirm that apoptosis sensitivities to all alkylphospholipids are dependent on lipid raft integrity (including appropriate cholesterol content) and SMS1-mediated sphingomyelin production in the lipid rafts.

Perifosine Accumulates in Lipid Rafts, Independent of Their Sphingomyelin Levels, but Internalization Depends on Sphingomyelin Synthesis

Because edelfosine was previously shown to accumulate in lipid rafts before its raft-mediated internalization (23–25), we investigated possible raft accumulation of perifosine, the only alkylphospholipid available in ^{14}C-labeled form. Figure 4 shows that, similar to edelfosine (26), [^{14}C]perifosine accumulated in lipid rafts to similar levels for S49 cells and $S49^{AR}$ and $S49^{siSMS1}$ cells that are deficient in sphingomyelin synthesis (Fig. 4A and C, *right*). In addition, when S49 cells were pretreated with bacterial sphingomyelinase to decrease sphingomyelin levels (Fig. 4B, *right*), there was no effect on the preference of perifosine to accumulate in rafts (Fig. 4B). Therefore, we conclude that the levels of sphingomyelin in lipid rafts are irrelevant for the accumulation of perifosine into these membrane microdomains.

Although the content of sphingomyelin was irrelevant, its synthesis (which has functions beyond simply contributing to raft structure; see Discussion) was important for the active cellular uptake of perifosine, as it was for edelfosine (25). Similar to [^{3}H]edelfosine (Fig. 5A), the time-dependent uptake of [^{14}C]perifosine at 37°C was decreased in SMS1–down-regulated cells ($S49^{AR}$ and $s49^{siSMS1}$) as compared with the parental S49 cells (Fig. 5B). Also, similar to edelfosine (23), internalization of [^{14}C]perifosine was dependent on lipid raft integrity because raft disruption of S49 cells with the cholesterol-sequestrating agents filipin and MβCD or with bSMase decreased the uptake of this alkylphospholipid (Fig. 5C).

Different Levels of Cellular Uptake of Perifosine Compared with Edelfosine

When comparing the uptake at 37°C of edelfosine with perifosine in more detail, we found that the perifosine taken up by S49 cells remained less persistently associated with (bound to) the cells than edelfosine (Fig. 6). This persistence of alkylphospholipid accumulation in the cells was shown by back-extraction with fatty acid–free bovine serum albumin (BSA), a technique that we used previously to analyze the internalization of exogenous lysophosphatidylcholine in cells (24). BSA will only back-extract the alkylphospholipid fraction that remains in the outer leaflet of the plasma membrane lipid bilayer, not the alkylphospholipid that has been internalized by spontaneous and/or protein-mediated transbilayer ''flipping'' or endocytosis. Figure 6A and B shows the relative amount of radiolabeled edelfosine that, after 10 min or 1 h incubation at 37°C,

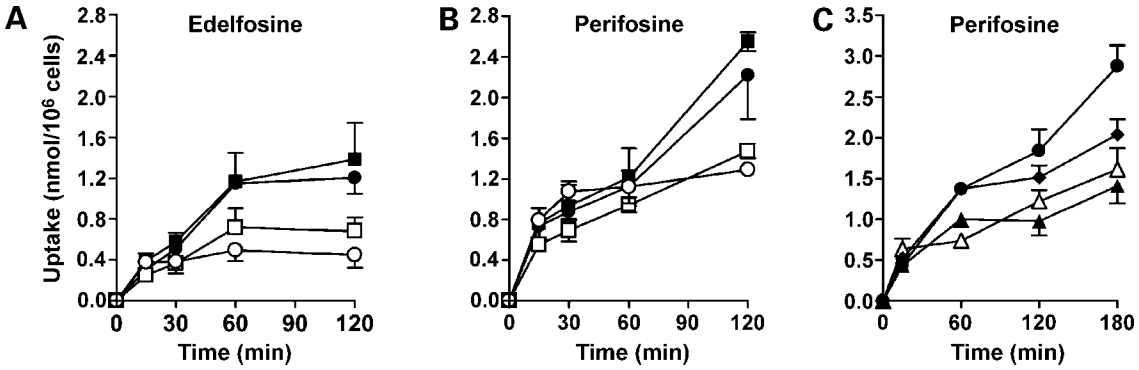

Figure 5. Time-dependent cellular uptake of edelfosine and perifosine; dependence on SMS1 expression (**A** and **B**) and lipid raft integrity (**C**). Cells were incubated at 37°C with [^{3}H]edelfosine (15 μmol/L, 0.02 μCi/mL; **A**) or [^{14}C]perifosine (20 μmol/L, 0.02 μCi/mL; **B** and **C**) for the times indicated and then washed with cold PBS and solubilized, and the radioactivity was counted. From this, the amount of uptake in nanomoles (±SD; $n = 4$) was calculated. Symbols for cells in **A** and **B**, S49, ●; $S49^{AR}$, ○; $S49^{mock}$; ■; $S49^{siSMS1}$, □. **C**, S49 cells remained untreated (●) or were pretreated (30 min) with filipin (1 μg/mL; ◆), methyl-β-cyclodextrin (2 mg/mL; △), or bacterial sphingomyelinase (150 milliunits/mL; ▲).

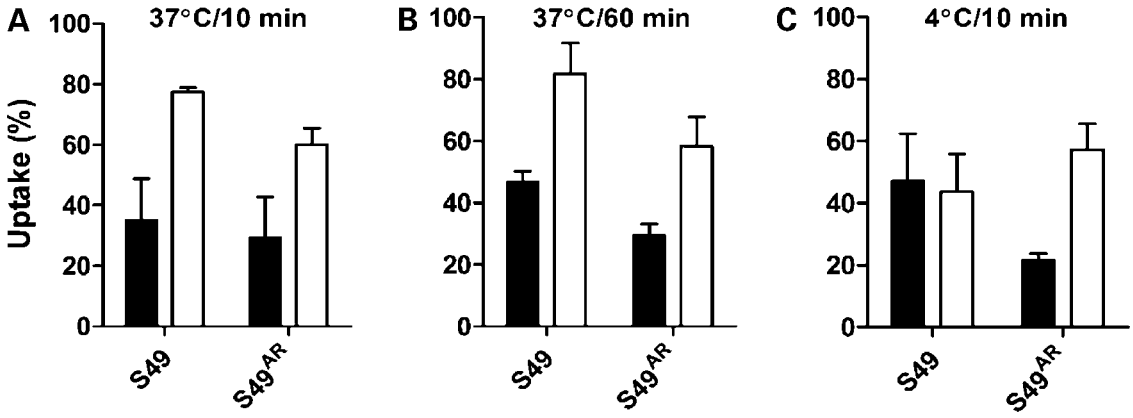

Figure 6. Fraction of the cellular uptake of edelfosine and perifosine that is resistant to BSA back-extraction. S49 and S49^{AR} cells were incubated at 37°C (**A** and **B**) or at 4°C (**C**) with [^{3}H]edelfosine (15 μmol/L, 0.02 μCi/mL; *open columns*) or [^{14}C-]perifosine (20 μmol/L, 0.02 μCi/mL; *closed columns*) for the times indicated (10 min, 1 h) and then washed thrice with cold PBS or with fatty acid–free BSA (1%). Data are expressed as the percentage of radioactivity left in the cells after BSA washing relative to PBS washing. Data are means of three experiments ± SD.

remains associated to cells after BSA back-extraction, which was a about 2-fold higher than for perifosine. These levels of internalized alkylphospholipid were higher in S49 cells than in S49^{AR} cells, which is explained by the lack of raft-mediated endocytosis in the latter cells (23, 25).

We next determined the persistent uptake at 4°C, a temperature that still allows spontaneous transbilayer flipping (24) but no active endocytic uptake. Figure 6C shows that the same percentage (about 45%) of edelfosine and perifosine remained associated to S49 cells after BSA back-extraction, suggesting that the internalization by spontaneous flipping is the same for the two alkylphospholipids. Intriguingly, however, from S49^{AR} cells, much more perifosine (80%) than edelfosine (45%) can be back-extracted under these low-temperature conditions, suggesting that in these alkylphospholipid-resistant cells, perifosine is less subject to trans-bilayer flipping than edelfosine for reasons that are not clear.

The most important conclusion from these data is that the cellular uptake of perifosine is less efficient than that of edelfosine, so that more perifosine than edelfosine remains located at the plasma membrane outer leaflet, available to BSA back-extraction. Although spontaneous membrane traversal (flipping) in S49 cells seems the same for the two compounds, temperature-dependent active uptake is higher for edelfosine than for perifosine.

Discussion

In this paper, we have shown that S49 lymphoma cells are sensitive to a class of structurally related synthetic alkylphospholipids, which comprises edelfosine, D-21805, erucylphosphocholine, and perifosine. The relative potency of these four anticancer agents to induce apoptosis in S49 cells differed and was individually correlated with their capacity to inhibit phosphatidylcholine synthesis in the cell. We showed for each of these compounds that this inhibition of phosphatidylcholine synthesis was a direct trigger for apoptosis induction because exogenous lysophosphatidylcholine, an alternative source for phosphatidylcholine production (through acylation inside the cell), rescued the cells from alkylphospholipid-induced apoptosis. For two of these compounds, (radiolabeled) edelfosine and perifosine, we showed that they accumulated in the detergent-resistant lipid raft fractions, and that the cellular uptake was impaired when the rafts were disrupted by cholesterol extraction (using MβCD) or when the synthesis of a major raft phospholipid, sphingomyelin, was down-regulated. These latter interventions protected the cells against apoptosis induction by all alkylphospholipids. It is therefore likely that each of these alkylphospholipids, after initial insertion in the outer leaflet of the plasma membrane lipid bilayer, accumulates in lipid rafts and, from there, is taken up by raft-mediated endocytosis, as we showed previously for edelfosine in more detail (23–25).

The differential efficacy of apoptosis induction by the four alkylphospholipids (Figs. 1 and 2B) is likely related to their different chemical structure (Fig. 1A). Edelfosine, the most effective one, contains a glycerol backbone with two ether-linked substituents: a long alkyl chain and a short *O*-methyl group, properties that allow its easy partitioning into lipid rafts, as argued before (23, 28, 29). In model membranes, hexadecylphosphocholine (miltefosine; lacking the glycerol backbone) was less miscible with sphingomyelin than edelfosine, yet stabilized sphingomyelin-cholesterol–rich ordered domains, similar to edelfosine (29). This is in line with our finding that perifosine, like edelfosine, accumulates in raft fractions, but possibly in weaker association with sphingomyelin. If true, it remains to be seen if this relates to the degree of cellular uptake, the route by which this occurs, and the apoptotic efficacy. The lower efficacy of perifosine to induce apoptosis cannot be ascribed to metabolic breakdown because the molecule remained essentially intact within the cell (30).

When comparing the most potent alkylphospholipid, edelfosine, with the least effective one, perifosine, we find

some similarities, but also differences in their behavior in cells. Both compounds accumulate in rafts, but they differ in the capacity to inhibit phosphatidylcholine synthesis. It is possible that different alkylphospholipids have differential inhibitory effects on the CTP:phosphocholine cytidylyltransferase per se. However, it is also possible that not every alkylphospholipid can reach this enzyme in the endoplasmic reticulum by the same route and to the same extent. In this regard, it is of interest that perifosine, after cellular uptake, can be much more easily back-extracted by BSA than edelfosine. This would suggest a less pronounced cellular internalization, possibly by a different route and/or by staying more in the cell periphery than edelfosine. The mechanism of cellular internalization and distribution of alkylphospholipid is probably also dependent on the cell type, as we previously observed that KB cells showed a remarkable high uptake and sensitivity for perifosine compared with the other squamous carcinoma cell lines A431 and HNXOE (30).

It is a remarkable finding that perifosine incorporation in lipid rafts is independent of sphingomyelin content. Neither inhibition of sphingomyelin synthesis by RNAi-mediated SMS1 down-regulation nor sphingomyelin breakdown by bacterial sphingomyelinase affected perifosine incorporation into the lipid raft fractions. Similar results were recently published for edelfosine (25). However, sphingomyelin synthesis was important for cellular uptake of perifosine and the subsequent induction of apoptosis because down-regulation of SMS1 by RNAi prevented these events, again, similar as we found for edelfosine (25). In this regard, it should be noted that SMS1 activity in the trans-Golgi (31) has important cell biological implications that goes beyond the mere production of sphingomyelin for nascent lipid rafts (25). SMS1 also produces diacylglycerol that activates protein kinase D, which is essential for anterograde vesicular trafficking toward the plasma membrane (32). We have argued that alkylphospholipid internalization by raft-dependent endocytosis may represent the retrograde route of constitutive lipid raft-vesicular cycling that may exist between the trans-Golgi, the plasma membrane, and the endosomal compartments (25).

Our results strengthen the idea that inhibition of phosphatidylcholine synthesis is a major insult to cells, which is apparently sufficient to initiate their apoptotic machinery, at least in S49 lymphoma cells. Not only edelfosine but also other alkylphospholipids can induce apoptosis in this way. How exactly phosphatidylcholine synthesis inhibition by alkylphospholipids leads to apoptosis is unknown. Rescue of cells from cell death by exogenous lysophosphatidylcholine, which is rapidly converted to phosphatidylcholine (24), suggests that a continuous phosphatidylcholine synthesis is crucial of cell survival. We have argued that a continuous phospholipid supply by vesicular trafficking might support a survival mechanism (33). In line with this speculative idea is that proper phosphatidylcholine synthesis may regulate PKB/Akt kinase activity, as it was shown that an inhibition in phosphatidylcholine synthesis precedes PKB/Akt inactivation (34). This might be a reason why perifosine and edelfosine inhibited PKB/Akt activation in various cell types (16, 35, 36). However, the precise mechanism by which this phosphatidylcholine synthesis inhibition connects in molecular terms to the initiation of the apoptotic machinery remains to be investigated. Also, future studies should reveal to what extent such a raft- and SMS1-dependent alkylphospholipid uptake mechanism applies to other types of (human) cancer cells.

Acknowledgments

Zentaris GmbH (Frankfurt, Germany) is acknowledged for providing compound D-21805, erucylphosphocholine, and (radiolabeled) perifosine.

References

1. Munder PG, Westphal O. Antitumoral and other biomedical activities of synthetic ether lysophospholipids. Chem Immunol 1990;49:206–35.

2. Unger C, Damenz W, Fleer EA, et al. Hexadecylphosphocholine, a new ether lipid analogue. Studies on the antineoplastic activity *in vitro* and *in vivo*. Acta Oncol 1989;28:213–7.

3. Mollinedo F, Fernandez-Luna JL, Gajate C, et al. Selective induction of apoptosis in cancer cells by the ether lipid ET-18-OCH3 (Edelfosine): molecular structure requirements, cellular uptake, and protection by Bcl-2 and Bcl-X(L). Cancer Res 1997;57:1320–8.

4. Ruiter GA, Zerp SF, Bartelink H, van Blitterswijk WJ, Verheij M. Alkyl-lysophospholipids activate the SAPK/JNK pathway and enhance radiation-induced apoptosis. Cancer Res 1999;59:2457–63.

5. Terwogt JM, Mandjes IA, Sindermann H, Beijnen JH, ten Bokkel Huinink WW. Phase II trial of topically applied miltefosine solution in patients with skin-metastasized breast cancer. Br J Cancer 1999;79:1158–61.

6. Dummer R, Roger J, Vogt T, et al. Topical application of hexadecylphosphocholine in patients with cutaneous lymphomas. Prog Exp Tumor Res 1992;34:160–9.

7. Berger MR, Sobottka S, Konstantinov SM, Eibl H. Erucylphosphocholine is the prototype of i.v. injectable alkylphosphocholines. Drugs Today (Barc) 1998;34 (Suppl. F):73–81.

8. Jendrossek V, Erdlenbruch B, Hunold A, Kugler W, Eibl H, Lakomek M. Erucylphosphocholine, a novel antineoplastic ether lipid, blocks growth and induces apoptosis in brain tumor cell lines *in vitro*. Int J Oncol 1999;14:15–22.

9. Handrick R, Rubel A, Faltin H, Eibl H, Belka C, Jendrossek V. Increased cytotoxicity of ionizing radiation in combination with membrane-targeted apoptosis modulators involves downregulation of protein kinase B/Akt-mediated survival-signaling. Radiother Oncol 2006;80:199–206.

10. Rubel A, Handrick R, Lindner LH, et al. The membrane targeted apoptosis modulators erucylphosphocholine and erucylphosphohomocholine increase the radiation response of human glioblastoma cell lines *in vitro*. Radiat Oncol 2006;1:6.

11. Dasmahapatra GP, Didolkar P, Alley MC, Ghosh S, Sausville EA, Roy KK. *In vitro* combination treatment with perifosine and UCN-01 demonstrates synergism against prostate (PC-3) and lung (A549) epithelial adenocarcinoma cell lines. Clin Cancer Res 2004;10:5242–52.

12. Li X, Luwor R, Lu Y, Liang K, Fan Z. Enhancement of antitumor activity of the anti-EGF receptor monoclonal antibody cetuximab/C225 by perifosine in PTEN-deficient cancer cells. Oncogene 2006;25:525–35.

13. Rahmani M, Reese E, Dai Y, et al. Coadministration of histone deacetylase inhibitors and perifosine synergistically induces apoptosis in human leukemia cells through Akt and ERK1/2 inactivation and the generation of ceramide and reactive oxygen species. Cancer Res 2005;65:2422–32.

14. Nyakern M, Cappellini A, Mantovani I, Martelli AM. Synergistic induction of apoptosis in human leukemia T cells by the Akt inhibitor perifosine and etoposide through activation of intrinsic and Fas-mediated extrinsic cell death pathways. Mol Cancer Ther 2006;5:1559–70.

15. Momota H, Nerio E, Holland EC. Perifosine inhibits multiple signaling

pathways in glial progenitors and cooperates with temozolomide to arrest cell proliferation in gliomas *in vivo*. Cancer Res 2005;65:7429 – 35.

16. Hideshima T, Catley L, Yasui H, et al. Perifosine, an oral bioactive novel alkylphospholipid, inhibits Akt and induces *in vitro* and *in vivo* cytotoxicity in human multiple myeloma cells. Blood 2006;107:4053 – 62.

17. Vink SR, Lagerwerf S, Mesman E, et al. Radiosensitization of squamous cell carcinoma by the alkylphospholipid perifosine in cell culture and xenografts. Clin Cancer Res 2006;12:1615 – 22.

18. Vink SR, Schellens JH, Beijnen JH, et al. Phase I and pharmacokinetic study of combined treatment with perifosine and radiation in patients with advanced solid tumours. Radiother Oncol 2006;80:207 – 13.

19. Vink SR, van Blitterswijk WJ, Schellens JH, Verheij M. Rationale and clinical application of alkylphospholipid analogues in combination with radiotherapy. Cancer Treat Rev 2007;33:191 – 202.

20. Ruiter GA, Verheij M, Zerp SF, van Blitterswijk WJ. Alkyl-lysophospholipids as anticancer agents and enhancers of radiation-induced apoptosis. Int J Radiat Oncol Biol Phys 2001;49:415 – 9.

21. Boggs KP, Rock CO, Jackwoski S. Lysophosphatidylcholine and 1-*O*-octadecyl-2-*O*-methyl-*rac*-glycero-3-phosphocholine inhibit the CDP-choline pathway of phosphatidylcholine synthesis at the CTP:phosphocholine cytidylyltransferase step. J Biol Chem 1995;270:7757 – 64.

22. Boggs KP, Rock CO, Jackwoski S. The antiproliferative effect of hexadecylphosphocholine toward HL60 cells is prevented by exogenous lysophosphatidylcholine. Biochim Biophys Acta 1998;1389:1 – 12.

23. Van der Luit AH, Budde M, Ruurs P, Verheij M, van Blitterswijk WJ. Alkyl-lysophospholipid accumulates in lipid rafts and induces apoptosis via raft-dependent endocytosis and inhibition of phosphatidylcholine synthesis. J Biol Chem 2002;277:39541 – 7.

24. Van der Luit AH, Budde M, Verheij M, van Blitterswijk WJ. Different modes of internalization of apoptotic alkyl-lysophospholipid and cell-rescuing lysophosphatidylcholine. Biochem J 2003;374:747 – 53.

25. Van der Luit AH, Budde M, Zerp S, et al. Resistance to alkyl-lysophospholipid – induced apoptosis due to downregulated sphingomyelin synthase 1 expression with consequent sphingomyelin- and cholesterol-deficiency in lipid rafts. Biochem J 2007;401:541 – 9.

26. Smets LA, Van Rooij H, Salomons GS. Signalling steps in apoptosis by ether lipids. Apoptosis 1999;4:419 – 27.

27. Nicoletti I, Migliorati G, Pagliacci MC, Grignani F, Riccardi C. A rapid and simple method for measuring thymocyte apoptosis by propidium iodide staining and flow cytometry. J Immunol Methods 1991;139:271 – 9.

28. Mattjus P, Bittman R, Slotte JP. Molecular interactions and lateral domain formation in monolayers containing cholesterol and phosphatidylcholines with acyl- or alkyl-linked C16 chains. Langmuir 1996;12:1284 – 90.

29. Heczkova B, Slotte JP. Effect of anti-tumor ether lipids on ordered domains in model membranes. FEBS Lett 2006;580:2471 – 6.

30. Vink SR, Schellens JH, van Blitterswijk WJ, Verheij M. Tumor and normal tissue pharmacokinetics of perifosine, an oral anti-cancer alkylphospholipid. Invest New Drugs 2005;23:279 – 86.

31. Huitema K, van den Dikkenberg J, Brouwers JFHM, Holthuis JCM. Identification of a family of animal sphingomyelin synthases. EMBO J 2004;23:33 – 44.

32. Bankaitis VA, Morris AJ. Lipids and the exocytotic machinery of eukariotic cells. Curr Opin Cell Biol 2003;15:389 – 95.

33. Van Blitterswijk WJ, van der Luit AH, Veldman RJ, Verheij M, Borst J. Ceramide: second messenger or modulator of membrane structure and dynamics? Biochem J 2003;369:199 – 211.

34. Kawada M, Masuda T, Ishizuka M, Takeuchi T. 15-Deoxyspergualin inhibits Akt kinase activation and phosphatidylcholine synthesis. J Biol Chem 2002;277:27765 – 71.

35. Ruiter GA, Zerp SF, Bartelink H, van Blitterswijk WJ, Verheij M. Anticancer alkyl-lysophospholipids inhibit the phosphatidylinositol 3-kinase-Akt/PKB survival pathway. Anticancer Drugs 2003;2:167 – 73.

36. Kondapaka SB, Singh SS, Dasmahapatra GP, Sausville EA, Roy KK. Perifosine, a novel alkylphospholipid, inhibits protein kinase B activation. Mol Cancer Ther 2003;2:1093 – 103.

Molecular Cancer Therapeutics

A new class of anticancer alkylphospholipids uses lipid rafts as membrane gateways to induce apoptosis in lymphoma cells

Arnold H. van der Luit, Stefan R. Vink, Jeffrey B. Klarenbeek, et al.

Mol Cancer Ther 2007;6:2337-2345.