Cancer Chemother Pharmacol (2002) 50: 71–79
DOI 10.1007/s00280-002-0440-8

ORIGINAL ARTICLE

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Structure-activity relationships of alkylphosphocholine derivatives: antineoplastic action on brain tumor cell lines in vitro

Received: 22 October 2001 / Accepted: 6 February 2002 / Published online: 5 June 2002

Abstract Erucylphosphocholine (ErPC) is a promising candidate for the treatment of human brain tumors. The aim of the present study was to investigate whether structural modifications of ErPC would improve its antineoplastic activity in vitro. The novel alkylphosphocholine (APC) derivatives docosenyl-(*cis*-10,11)-phosphocholine, tricosenyl-(*cis*-12,13)-phosphocholine, heneicosenyl-(*cis*-12,13)-phosphocholine and erucyl-*N*,*N*,*N*-trimethylpropanolaminophosphate all reduced cell growth and viability of rat and human astrocytoma/glioblastoma (AC/GBM) cell lines (C6, T98G, U87MG, A172) and had improved antineoplastic activity when compared to the prototypical APC hexadecylphosphocholine (HePC). However, the four cell lines differed in their sensitivity to the APC derivatives. A172 cells were most sensitive to their cytostatic action and T98G cells to their cytotoxic action. The LC_{50} values for T98G cells after a 72-h exposure to the novel derivatives varied between 25 and 54 μM compared to 45 ± 8.1 μM for ErPC. Complete killing of T98G cells was obtained with all derivatives at 90 μM. Structural modifications of the chain length of the alcohol moiety as well as changing the position of the double bond within the alkyl chain improved cytotoxicity of the APC in C6 and A172 cells and to a lesser extent in T98G cells, whereas U87MG cells showed almost similar sensitivities to the novel drugs and ErPC. Increasing the distance between the phosphorus and nitrogen atoms within the polar phosphocholine group did not alter antineoplastic activity but modified physicochemical characteristics, e.g. increased the solubility in water. In a similar manner to ErPC, all derivatives induced growth arrest in the G_2/M phase of the cell cycle and apoptotic cell death. Importantly, none of the derivatives showed hemolytic activity. As there was no clear superiority of any of the novel derivatives, ErPC remains the leading APC derivative for future clinical trials in brain tumor chemotherapy.

Keywords Alkylphosphocholines · Erucylphosphocholine · Glioblastoma · Structure-activity relationship · Apoptosis

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Introduction

Despite some progress in the treatment of several brain tumors such as medulloblastoma or primitive neuroectodermal tumors, the overall prognosis of patients with malignant gliomas has not been improved significantly [7, 17]. Glioblastoma (GBM) is almost uniformly fatal with only a few patients surviving longer than 2 years [9]. Two major problems impair the success of chemotherapy. First, the delivery of sufficient amounts of most antineoplastic drugs into the brain tissue is prevented by the blood-brain barrier. Second, high-grade gliomas are often characterized by high intrinsic chemoresistance. Therefore, the use of lipophilic antineoplastic drugs with efficient penetration across the blood-brain barrier, as well as drugs with new mechanisms of action bypassing chemoresistance, could basically improve the chemotherapy of malignant brain tumors.

Alkylphosphocholines (APC) represent a novel class of lipophilic ether lipids with promising antitumor activity in vitro and in vivo [8, 12, 18, 20, 21, 32]. They were derived from the antineoplastic alkyllysophospholipids (ALP) that are related to natural lysophospholipids. The ALP derivative 1-*O*-octadecyl-2-*O*-methyl-*rac*-glycero-3-phosphocholine (Et-18-OCH_3) shows high antineoplastic

activity against various tumor cell lines in vitro including brain tumor cell lines [2, 28, 33]. However, due to their rapid biotransformation in vivo, cytotoxic drug concentrations cannot be achieved in tissues and organs [1, 27].

Eibl and coworkers have identified the APC as the minimal structural requirement for antineoplastic activity of the ALP [31, 34]. APC are phosphocholine esters of long-chain aliphatic alcohols of different chain length. Due to the lack of the glycerol moiety, APC are not metabolized by phospholipases and therefore accumulate in organs and tissues in vivo [14, 24]. These compounds, unlike conventional DNA-damaging agents, are membrane-targeted drugs primarily modulating membrane lipid composition and phospholipid metabolism [5, 30, 36]. Inhibition of mitogenic signal transduction and induction of apoptosis contribute to the antineoplastic action of these compounds [4, 10, 16, 26, 28, 37]. In contrast to DNA-damaging drugs, APC show no myelotoxicity and even stimulate growth of hematopoietic progenitor cells [6, 15].

Erucylphosphocholine (ErPC) is the first APC derivative which can be given intravenously [11, 14]. It exerts antineoplastic effects in vivo against methylnitrosurea-induced mammary carcinoma in the rat [3, 24] and against brain tumor cell lines in vitro [13]. In these studies, ErPC has turned out to be more active than the prototypical APC derivative hexadecylphosphocholine (HePC). It modulates specific intracellular signaling pathways leading to growth arrest in the G_2/M phase of the cell cycle and the induction of programmed cell death even in chemoresistant astrocytoma/glioblastoma (AC/GBM) cell lines [18, 19]. Pharmacokinetic experiments in healthy and tumor-bearing rats have revealed that ErPC crosses the blood-brain barrier and accumulates in the brain, reaching concentrations sufficient to kill human GBM cell lines in vitro. Moreover, no severe toxic side effects have been observed at these concentrations [14]. Thus, ErPC is the most promising ether lipid for the treatment of malignant gliomas.

The aim of the present study was to identify the structural requirements for efficient antineoplastic action of APC against brain tumor cells in vitro. The structural variations of the ErPC molecule included the position of the double bond, the chain length of the alcohol moiety and the hydrophilic phosphocholine group. The position of the double bond and the chain length influenced the cytotoxic effects of APC in C6, A172 and, to a lesser extent, T98G cells. In a similar manner to ErPC, all APC derivatives induced growth arrest in the G_2/M phase of the cell cycle and apoptotic cell death. None of them showed hemolytic activity. Based on their high antineoplastic activity or their favorable physicochemical properties, docosenyl-(*cis*-10,12)-phosphocholine (C22:1), heneicosenyl-(*cis*-12,13)-phosphocholine (C21:1) and erucyl-*N*,*N*,*N*-trimethylpropanolaminophosphate ($ErPC_3$) could be recommended for evaluation in animal experiments. However, there was no clear superiority of any of the novel APC derivatives regarding their antineoplastic action in the AC/GBM cell lines tested. Therefore, ErPC remains the APC derivative of choice for future clinical trials in brain tumor chemotherapy.

Materials and methods

Chemicals and drugs

All APC derivatives were synthesized by H. Eibl, Max Planck-Institute for Biophysical Chemistry, Göttingen, Germany. The chemical modifications of the compounds consisted of (a) varying the length of the alkyl chain by the addition or deletion of one CH_2 group in the alkyl chain (C23:1 and C21:1), (b) changing the position of the double bond to *cis*-10,11 (C22:1) and (c) increasing the distance between the phosphorus and nitrogen atoms by one CH_2 group in the choline moiety ($ErPC_3$) (Fig. 1; Table 1). In addition, HePC, the prototypical APC without a double bond, was used as a reference.

The drugs were dissolved in 200 µl ethanol and diluted with RPMI-1640 medium supplemented with 10% fetal calf serum to a concentration of 10 or 20 m*M* (stock solution). The final ethanol concentration in tissue culture experiments was below 0.1%. For hemolysis experiments, APC derivatives were dissolved in 200 or 500 µl ethanol and diluted with PBS to a concentration of 20 or 50 m*M*.

The cell culture medium and supplements were purchased from Seromed (Berlin, Germany). The WST-1 and 5-bromo-2′-deoxyuridine (BrdU) test were from Roche Molecular Biochemicals (Mannheim, Germany). All other chemicals were purchased from Sigma-Aldrich (Deisenhofen, Germany).

Cell lines

The C6 rat glioma cell line as well as the human AC/GBM cell lines A172, U87MG and T98G were obtained from ATCC (Rockville, Md.). All tumor cell lines were adapted to RPMI-1640 medium containing 2 m*M* glutamine and 2 g/l sodium bicarbonate (PAA, Linz, Austria) supplemented with 10% (v/v) heat-inactivated fetal bovine serum and 100 U/ml penicillin and 100 µg/ml streptomycin (Life Technologies, Karlsruhe, Germany). All cell lines were grown as monolayers in tissue culture flasks in a humidified atmosphere of 5% CO_2/95% air at 37°C.

Determination of cell viability and proliferation

Cells were seeded into 96-well plates (1×10^3 cells/well) and drugs diluted in culture medium were added to the cells 24 h after plating. Drug sensitivity was assessed with cells after continuous exposure to the drug of interest for 24–72 h by the WST-1 test as described elsewhere [18]. The LC_{50} represents the drug concentration causing a reduction in the number of viable cells to 50% of the number of

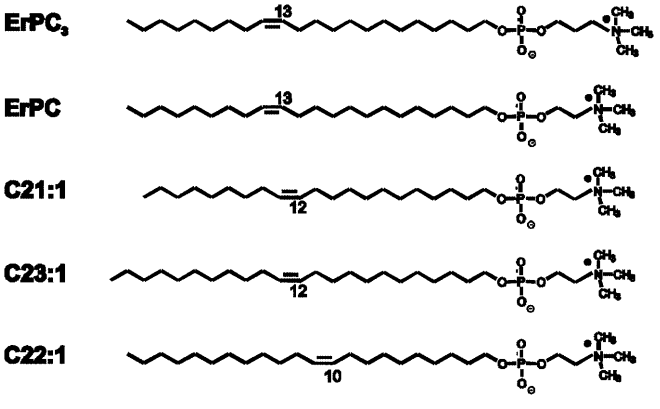


Fig. 1. Chemical structures of APC derivatives

Table 1. Structural characteristics and short names of ErPC derivatives

Name	Short name	Number of *cis* double bonds	Location of double bond	Alkyl chain (C-atoms)	Polar head
Docosenyl-(*cis*-10,11)-phosphocholine	C22:1	1	*Cis*-10,11	22	Phosphocholine
Tricosenyl-(*cis*-12,13)-phosphocholine	C23:1	1	*Cis*-12,13	23	Phosphocholine
Heneicosenyl-(*cis*-12,13)-phosphocholine	C21:1	1	*Cis*-12, 13	21	Phosphocholine
Erucyl-*N*,*N*,*N*-trimethyl-propanolaminophosphate	$ErPC_3$	1	*Cis*-13,14	22	*N*,*N*,*N*-Trimethylpropanolaminophosphate
Erucylphosphocholine	ErPC	1	*Cis*-13,14	22	Phosphocholine
Hexadecylphosphocholine	HePC	0	–	16	Phosphocholine

untreated control cells. Additionally, viability was determined by trypan blue exclusion. For this purpose, cells were harvested by trypsinization, dissolved in PBS, and an aliquot was stained for 1 min in the presence of 0.1% (w/v) trypan blue in PBS.

Cell proliferation was determined measuring BrdU incorporation into the DNA of proliferating cells using a colorimetric cell proliferation kit as described in the manufacturer's protocol (Roche Molecular Biochemicals, Mannheim, Germany). Absorbance at 370 nm (λ reference 492 nm) was determined in a Dynatech microplate reader MR600 (Dynatech Laboratories, Chantilly, Va.). The IC_{50} values are the drug concentrations causing a 50% reduction in BrdU incorporation when compared to that of untreated control cells.

Cell morphology

Cells were cultured for different times in medium alone or in medium supplemented with different concentrations of the APC. The morphology of the cells was evaluated by polarization light microscopy of native cells and by light microscopy of cells fixed in methanol and stained with Wright staining solution (0.3% w/v in methanol, pH 6.8).

Cell cycle analysis and quantification of apoptosis

Cells were harvested by trypsinization and washed twice with PBS. The cells (1×10^6/ml) were incubated in a hypotonic fluorochrome solution (50 µg/ml propidium iodide (PI) in 0.1% sodium citrate plus 0.1% Triton X-100) for 60 min at room temperature in the dark and subsequently submitted to FACS analysis [29]. PI fluorescence of individual cells after excitation by a 488 nm Argon laser was analyzed (640 nm long-pass filter) using a FACScan flow cytometer and CELLQuest and Modfit software (Becton Dickinson, Mountain View, Calif.).

Quantification of hemolysis

Blood from healthy donors was incubated with 2 or 5 m*M* of the APC derivatives. Blood incubated with the same concentrations of the solvent (4% or 10% ethanol) as well as erythrocytes and serum of untreated blood samples were used as controls. Aliquots were taken after 1, 5, 10 or 30 min of incubation. The samples were centrifuged to remove intact erythrocytes and the serum was used for the quantification of hemoglobin released during incubation. For this purpose, 20 µl of the serum was incubated with 5 ml of a solution for hemoglobin determination (Merck, Darmstadt, Germany) and the amount of hemoglobin was quantified by photometric detection at 546 nm.

Statistical evaluation

Experiments were performed in triplicate and the results are expressed as means ± SD except as indicated otherwise. The significance of differences in the data was analyzed using Student's *t*-test.

Table 2. IC_{50} values (µ*M*) in AC/GBM cell lines after 72 h of treatment. IC_{50} values are the drug concentrations that caused a reduction in the number of proliferating cells to 50% of the number of control cells as determined by BrdU incorporation. The values shown are means ± SD of three independent experiments. For the chemical structure of the derivatives refer to Table 1 and Fig. 1

Derivative	C6	U87MG	A172	T98G
C21:1	29 ± 2.3	103 ± 6.0	18 ± 5.0	31 ± 6.6
C23:1	48 ± 3.2	98 ± 6.4	24 ± 2.8	58 ± 15.7
C22:1	40 ± 8.7	104 ± 7.5	19 ± 0.6	44 ± 16.2
ErPC	76 ± 2.6	89 ± 4.6	36 ± 2.1	37 ± 1.0
$ErPC_3$	78 ± 3.8	79 ± 4.0	29 ± 2.3	35 ± 0.7
HePC	> 150	> 150	40 ± 0.8	71 ± 11.0

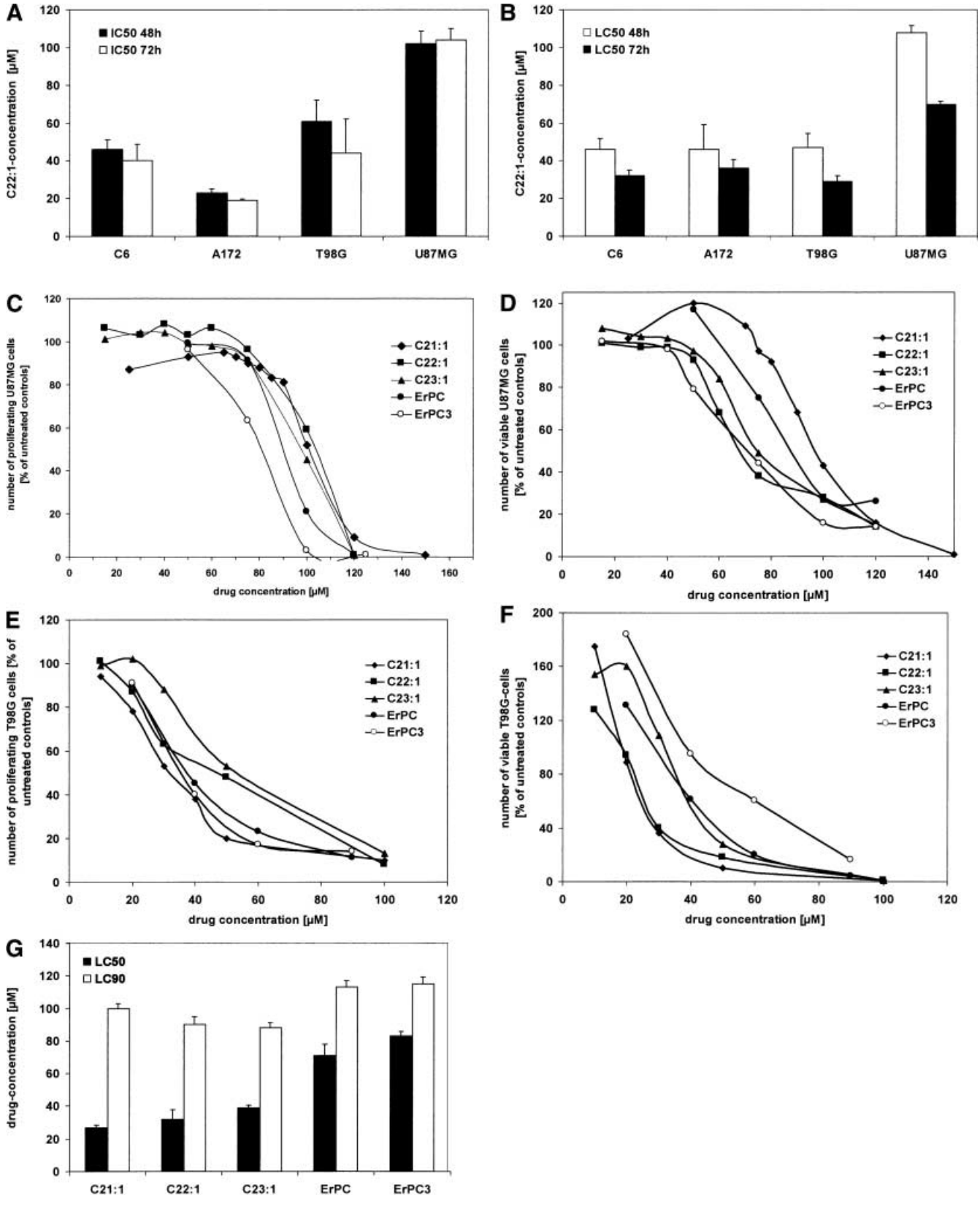
Table 3. LC_{50} values (µ*M*) in AC/GBM cell lines after 72 h of treatment. LC_{50} values are the drug concentrations that caused a reduction in the number of viable cells to 50% of the number of control cells as determined by the WST-1 test. The values shown are means ± SD of three independent experiments. For the chemical structure of the derivatives refer to Table 1 and Fig. 1

Derivative	C6	U87MG	A172	T98G
C21:1	27 ± 1.5	96 ± 1.5	29 ± 5.5	25 ± 1.0
C23:1	39 ± 1.4	77 ± 11.0	46 ± 2.9	43 ± 4.0
C22:1	32 ± 5.7	70 ± 2.5	36 ± 4.9	29 ± 3.2
ErPC	71 ± 6.9	89 ± 2.1	67 ± 3.2	45 ± 8.1
$ErPC_3$	83 ± 2.6	69 ± 2.1	66 ± 3.5	58 ± 8.0
HePC	145 ± 7.1	> 150	91 ± 2.1	89 ± 16.2

Results

AC/GBM cell lines differ in their sensitivity to APC derivatives

All APC derivatives exerted concentration-dependent cytostatic and cytotoxic effects on rat glioma C6 cells and the human AC/GBM cell lines A172, T98G and U87MG (Tables 2 and 3). The cytostatic and cytotoxic effects of the APC were maximal after 48 to 72 h (Fig. 2A, B). The cell lines differed in their sensitivity to the ether lipids with U87MG cells showing the lowest sensitivity to all of the derivatives tested (Tables 2 and 3; Fig. 2A, B). None



of the drugs inhibited proliferation of U87MG cells at concentrations below 55 µ*M* and complete inhibition required 100–120 µ*M* (Fig. 2C). Cytotoxicity was only observed at drug concentrations of ≥55 µ*M*, and drug concentrations ≥120 µ*M* were required for complete cell death (Fig. 2D). In contrast, T98G and A172 cells

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Fig. 2A–G. AC/GBM cell lines vary in their sensitivity to treatment with APC. **A**, **B** C6, A172, T98G and U87MG cells were grown in the presence of increasing concentrations of C22:1 for 48 and 72 h. **A** Cell proliferation was determined in terms of BrdU incorporation. IC_{50} values represent the drug concentrations that caused a 50% reduction in the number of proliferating cells (means ± SD, $n = 3$). **B** Cell viability was determined by the WST-1 test. The LC_{50} values represent the drug concentrations that caused a 50% reduction in the number of viable cells (means ± SD, $n = 3$). **C–F** Inhibition curves for the different APC derivatives (means, $n = 3$; SD omitted for clarity). U87MG cells (**C**, **D**) and T98G cells (**E**, **F**) were cultured for 72 h in the presence of increasing concentrations of the indicated drugs. Cell proliferation (**C**, **E**) and cell viability (**D**, **F**) were determined in terms of BrdU incorporation and the WST-1 test, respectively. **G** C6 cells were grown for 72 h in the presence of the indicated drugs. Cell viability was determined by the WST-1 test. LC_{50} and LC_{90} values represent the drug concentrations needed for a 50% or 90% reduction in the number of viable cells (means ± SD, $n = 3$)

turned out to be very sensitive to all APC derivatives (Tables 2 and 3; Fig. 2A, B). Antiproliferative and cytotoxic effects against these cells were observed at concentrations as low as 20–40 μ*M* and were maximal at 80–100 μ*M*, as shown for T98G cells in Fig. 2E, F.

C6 rat glioma cells differed in their sensitivity to the APC derivatives and turned out to be more sensitive to C21:1, C22:1 and C23:1 than to ErPC (Tables 2 and 3; Fig. 2G). Since the derivatives differed in the slope of their inhibition curves, the LC_{90} values were more similar than the LC_{50} values (Fig. 2G).

An increase in WST-1 reduction after treatment of U87MG and C6 cells with subtoxic concentrations of ErPC or C21:1 (10–20%) as well as after treatment of T98G or A172 cells with low concentrations of all APC derivatives tested (20–80%) was observed when compared to untreated control cells (Fig. 2D, F). This might be indicative of increased viability of the cells at low APC concentrations. In contrast, we were not able to detect a mitogenic effect of low APC concentrations on AC/GBM cells since the numbers of BrdU-incorporating cells was not increased at the same time (Fig. 2C, E, and data not shown).

Influence of structural variations on antineoplastic effects of APC derivatives

Structural modifications of the chain length of the alcohol moiety as well as changing the position of the double bond within the alkyl chain influenced the antineoplastic effects of the novel drugs in C6 and A172 cells and to a lesser extent in T98G cells, whereas the cytostatic and cytotoxic effects of the novel derivatives were almost similar in U87MG cells when compared to ErPC (Fig. 2C, D).

Comparing the derivatives with different chain lengths, derivatives with an alkyl chain length of 21, 22 or 23 carbon atoms were more effective than HePC, the C16:0 derivative. In C6, A172 and T98G cells the IC_{50} values were about twofold higher for HePC than for ErPC (Table 2). C21:1 and C23:1 exerted 2.3- to 2.6-fold stronger antiproliferative and cytotoxic effects than ErPC in C6 and A172 cells (Tables 2and 3, Fig. 3A and B). In addition, C21:1 turned out to be more cytotoxic than ErPC to T98G cells (Table 3, Fig. 2G).

The position of the double bond also strongly influenced the antineoplastic activity of the compounds. Comparing the LC_{50} values of C22:1 (*cis*-10,11) and ErPC (*cis*-13,14), C22:1 was significantly more cytotoxic to all four cell lines than ErPC (Table 3). Evaluation of cell viability after 48 h of treatment by the WST-1 test and by trypan blue exclusion gave similar results (Fig. 3C, D). Comparing C22:1 with ErPC, the LC_{50} values were decreased by factors of 1.6, 1.9 and 2.2 for T98G, A172 and C6 cells, respectively (Table 3). In addition, C6 and A172 cells were more sensitive to the antiproliferative action of C22:1 (Table 2).

However, modification of the polar phosphocholine group did not significantly alter the antineoplastic activity of the drug. All cell lines tested showed similar sensitivities to both ErPC and $ErPC_3$. No significant differences in the LC_{50} and IC_{50} values were observed (Tables 2 and 3). A putative advantage of $ErPC_3$ might be its altered physical properties with increased solubility in aqueous solutions (data not shown).

Toxicity and mechanism of action of the novel APC derivatives

Since the use of HePC was restricted by hemolytic effects, the release of hemoglobin from the blood of healthy donors was analyzed in an in vitro assay (Table 4). ErPC and its structural derivatives did not induce significant hemolysis at a concentration of 2 m*M*. The increased hemolysis at the higher concentration of 5 m*M* was due to the high concentration of the solvent (EtOH). No significant differences in the extent of hemolysis were observed between ErPC and the novel APC derivatives tested.

In previous work, we have shown that ErPC induces growth arrest of cells in the G_2/M phase of the cell cycle, bi- and multinuclear cell formation and apoptotic cell death [18]. Therefore, we sought to determine whether the novel derivatives share this mechanism of action with ErPC. The morphological evaluation of C6 cells by light microscopy showed a normal bipolar cell body with one nucleus in untreated cells (Fig. 4A). As under ErPC, treatment of C6 cells with 10–30 μ*M* of the novel APC derivatives induced bi- and multinuclear cell formation after 24 and 48 h (Fig. 4B). At higher drug concentrations (30–100 μ*M*) the APC derivatives uniformly induced cell rounding, membrane blebbing and the formation of apoptotic bodies indicative of the induction of apoptosis (Fig. 4C). To get further evidence for the induction of apoptosis we performed FACS analyses on C6 cells stained with PI in a hypotonic sodium citrate solution. Depending on the concentration, the novel APC derivatives also induced growth arrest of C6 cells in

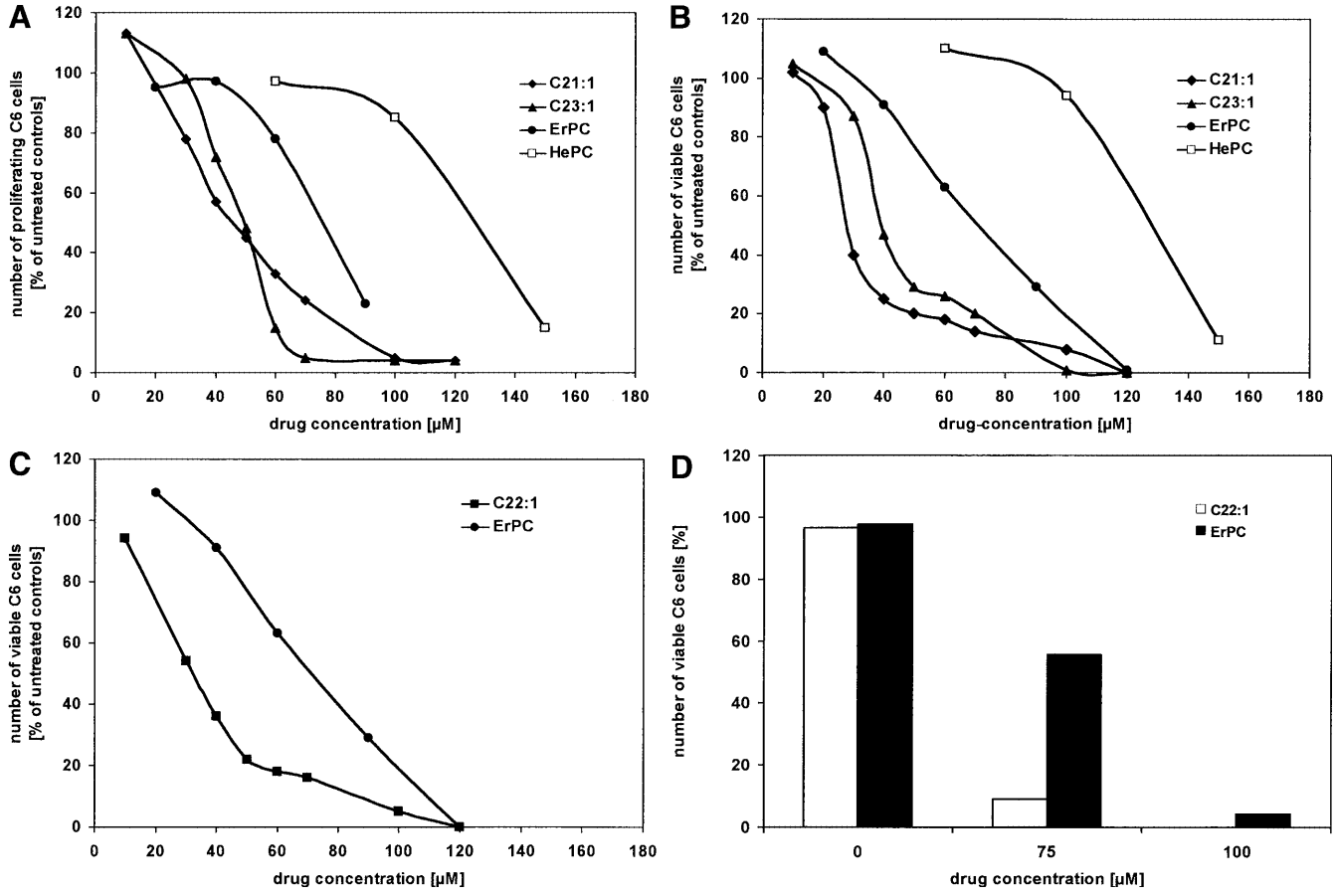


Fig. 3A–D. Structural variations influence antineoplastic activity of APC in AC/GBM cell lines. C6 cells were cultured for 72 h (**A**, **B**) or 48 h (**C**, **D**) in the presence of increasing concentrations of the indicated drugs. Cell proliferation (**A**) and cell viability (**B–D**) were determined in terms of BrdU incorporation (**A**), the WST-1 test (**B**, **C**) and trypan blue exclusion (**D**), respectively. **A–C** Inhibition curves for the different APC derivatives (means $n = 3$; SD omitted for clarity); **D** percentage of viable cells of one representative of three experiments giving similar results. Cells cultured in medium had viabilities of 94–99%

the G_2/M phase of the cell cycle as well as apoptotic cell death (Fig. 4D–F). In cells treated with medium alone, only 11–15% of the cells were found in the G_2/M phase of the cell cycle (region M4) in contrast to 30% after 48 h of treatment with 30 μ*M* C23:1 (Fig. 4D, E). At 100 μ*M* C23:1 about 50% of the cells were detected in the subG_1 peak (region M1) representing apoptotic cells (Fig. 4F).

The sensitivity of the cells to treatment with APC was dependent on their sensitivity to APC-induced apoptosis. In U87MG cells, which were rather insensitive to APC treatment, drug concentrations of 100 μ*M* were needed to induce 10–30% apoptosis within 48 h (data not shown). In contrast, 20 μ*M* of the APC was sufficient to induce significant apoptosis in the most sensitive T98G cells within 48 h of treatment. Again, T98G cells turned out to be most sensitive to C22:1 and C21:1, whereas increased concentrations of C23:1 and ErPC were needed to induce the same apoptotic response (Fig. 4G).

Discussion

ErPC is a promising APC derivative with a substantial antineoplastic action in brain tumor cell lines in vitro. Here, the antineoplastic activity of novel structural APC derivatives on brain tumor cell lines in vitro is described for the first time. The extent of the cytostatic and cytotoxic effects of these derivatives depended on the tumor cell line investigated. The human AC/GBM cell lines T98G and A172 were more sensitive to all APC derivatives tested than human U87MG cells and the rat glioma cell line C6. This is consistent with earlier observations on the action of ErPC [18]. These differences in the sensitivities might have been due to differences in drug uptake, the activity of phospholipid-metabolizing enzymes or the constitutive activity of key signaling molecules such as protein kinase C [35].

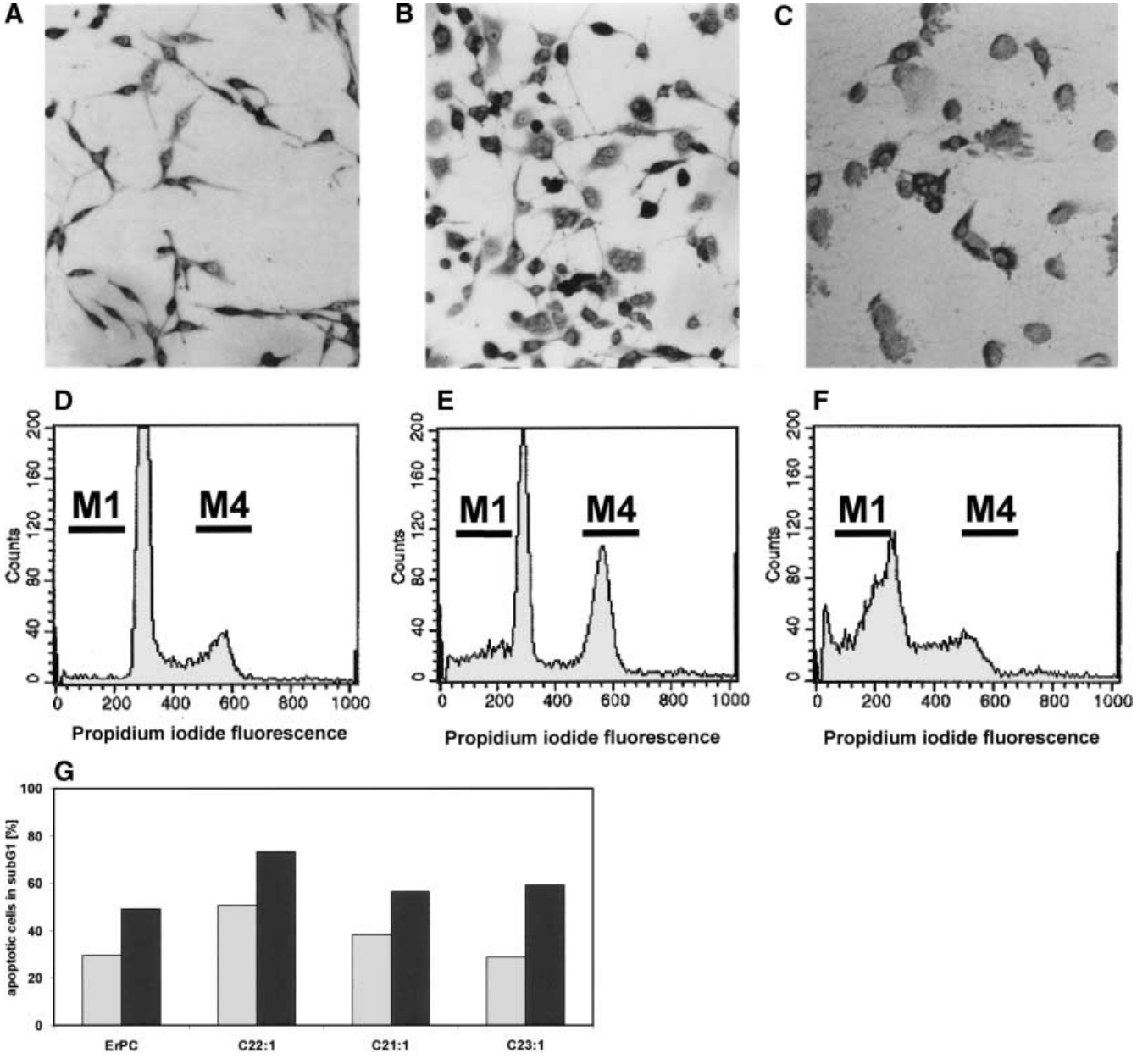
Analysis of the structural requirements for a strong antineoplastic effect in AC/GBM cells showed both the chain length of the alcohol moiety and the double bond to be important for the activity of the APC. HePC, the APC derivative with a 16 carbon atom alcohol moiety without a double bond (C16:0) was less effective than ErPC, whereas the C21:1, C22:1 and C23:1 derivatives turned out to be even more effective than ErPC in some of the cell lines. In A172 and C6 cells, LC_{50} and IC_{50} values of C21:1 and C22:1 were decreased by factors of

Table 4. Hemolytic activities of ErPC derivatives. Blood from healthy volunteers was treated with the indicated concentrations of the APC or the solvent (ethanol). After 30 min, the hemoglobin content of the serum was determined by an in vitro assay. Whole blood and serum of untreated blood samples were used as positive control (100% hemolysis) and negative control (0% hemolysis), respectively. Values shown are means of three independent experiments (SD $< 5\%$)

Drug	Hemolysis (% of complete hemolysis)	
	At 2 m*M* of the drug	At 5 m*M* of the drug
Solvent	1.3	35.0
ErPC	1.6	34.0
$ErPC_3$	1.1	36.5
C22:1	1.1	39.7
C21:1	1.3	41.8
C23:1	3.2	31.4

1.9–2.6 when compared to ErPC. Since in C21:1 and C23:1 the structural modification of the chain length of the alcohol moiety was combined with a switch of the

Fig. 4A–G. APC derivatives induce bi- and multinucleation, G_2/M arrest and apoptotic cell death. C6 cells were cultured in medium or in the presence of the indicated concentrations of C21:1 or C23:1. **A–C** Morphology was determined by Wright staining of fixed C6 cells after 48 h of treatment with (**A**) medium, (**B**) 30 μ*M* C21:1 and (**C**) 50 μ*M* C21:1. **D–F** FACS analyses of PI-stained C6 cells after 48 h of treatment with (**D**) medium, (**E**) 30 μ*M* C23:1 and (**F**) 100 μ*M* C23:1. *M1* and *M4* indicate the sub-G_1 peak (apoptotic cells) and the G_2/M phase of the cell cycle, respectively. **G** T98G cells were cultured for 48 h in medium with 20 (*white bars*) or 40 μ*M* (*black bars*) C21:1, C22:1, C23:1, or ErPC. Apoptosis was determined by FACS analysis of PI-stained cells. The data shown are from one representative of two experiments giving similar results



double bond position (*cis*-12,13 instead of *cis*-13,14), increased antineoplastic activity might be the sum of the favorable effects of the two variations.

The strongest antineoplastic activity was obtained with an alcohol moiety consisting of 21 to 23 carbon atoms and a *cis*-double bond at position 10,11 or 12,13 or 13,14. Our findings are consistent with earlier observations that the antineoplastic activity of APC depends on the chain length of the alkyl moiety [31]. Similarly, the antineoplastic activity against leukemic cells varies between different cell lines and the novel intravenously injectable derivatives have increased antileukemic activity compared to HePC [23]. In addition, the C22 derivatives ErPC and $ErPC_3$ are able to reduce the growth of human bladder carcinoma cell lines to a higher extent than derivatives with a shorter alkyl chain such as HePC [21]. It has also to be taken into account that structural variations influence the biodistribution of the drugs [24]. Therefore, in vivo activity cannot be concluded from in vitro data since tissue concentrations in brain tumors may not correlate with antineoplastic activity. Moreover, increasing the chain length of the hydrophilic phosphocholine group did not alter the antineoplastic activity of ErPC but modified its physical characteristics. The higher solubility of $ErPC_3$ in aqueous solutions compared to ErPC simplifies intravenous administration in vivo.

At subtoxic concentrations C21:1 and ErPC increased the viability of U87MG cells by 10–20%. This is consistent with earlier reports of increased viable cell counts in human tumor cell lines treated with low concentrations of HePC [34]. The authors assumed that at subtoxic doses APC might have mitogenic effects, presumably mediated through activation of protein kinase C and/or calcium mobilization from intracellular calcium stores [25, 35]. However, in the present study no increase in AC/GBM cell proliferation could be detected at subtoxic APC concentrations. On the other hand, ErPC-induced apoptosis has been shown to involve production of reactive oxygen species (ROS). These radicals by themselves are able to reduce WST-1. Therefore, in cell lines such as T98G that are highly sensitive to APC-induced apoptosis, the strongly increased WST-1 reduction at subtoxic APC doses could be due at least in part to the onset of ROS production during APC-induced apoptosis [19].

Hemolysis is a major toxic side effect of HePC. It has been shown that ErPC, a derivative with a monounsaturated alcohol chain, can be administered intravenously without hemolysis [14]. In accordance with this, none of the novel APC derivatives with a double bond tested in the present study induced hemolysis. Since these drugs can be given intravenously they may show reduced gastrointestinal toxicity and higher plasma concentrations in vivo [23]. Therefore, the double bond not only improved antineoplastic activity but in addition reduced toxic effects compared to HePC.

Investigation of the mechanism of action showed that, in a similar manner to ErPC, the novel APC derivatives induced growth arrest in the G_2/M phase of the cell cycle, bi- and multinuclear cell formation and apoptotic cell death in AC/GBM cells. The effects depended on the drug concentration and corresponded to the sensitivity of a given cell line to the respective derivative. T98G and A172 which were highly sensitive to the cytotoxic action of the APC showed extensive apoptotic cell death after APC treatment while the insensitive U87MG cells turned out to be rather resistant to APC-induced apoptosis. These results imply that the cytotoxicity of the APC correlated with their ability to induce apoptosis and that the sensitivity of a given cell line to APC treatment was dependent on its sensitivity to APC-induced apoptosis. Our results corroborate earlier findings that induction of apoptosis constitutes a common mechanism of the cytotoxic action of APC [16, 18, 22] and the structurally related ALP [10, 28].

In conclusion, the novel APC derivatives showed improved antineoplastic activity in AC/GBM cell lines and reduced toxicity when compared to the prototypical APC HePC. Apoptosis contributes to the cytotoxic action of these compounds. Structural requirements for strong antineoplastic activity against AC/GBM cell lines were a 21–23 carbon atom alkyl chain and a double bond in *cis*-10,11, *cis*-12,13 or *cis*-13,14 of the alkyl chain allowing intravenous administration. Based on the strong antineoplastic activity of C22:1 and C21:1 and the favorable physicochemical properties of $ErPC_3$, these derivatives could be recommended for evaluation in animal experiments. However, given that (1) the variations in the antineoplastic activity between different AC/GBM cell lines were as important as between the structural derivatives, (2) the overall antiproliferative and cytotoxic effects of the novel drugs did not show clear superiority compared to ErPC and (3) all pharmacological investigations focused on ErPC, ErPC remains the APC derivative of choice for future clinical trials in brain tumor therapy.

Acknowledgements Supported by a grant from the Deutsche Krebshilfe (10-1554-ErII) and the Fortune Program, Tübingen (768-0-0).

References

1. Arnold B, Reuther R, Weltzien HU (1978) Distribution and metabolism of synthetic alkyl analogues of lysophosphatidylcholine in mice. Biochim Biophys Acta 530:47
2. Berdel WE, Greiner E, Fink U, Zänker KS, Stavrou D, Trappe A, Fahlbusch R, Reichert A, Rastetter J (1984) Cytotoxic effects of alkyl-lysophospholipids in human brain tumor cells. Oncology 41:140
3. Berger MR, Eibl H (1992) Erucylphosphocholine is a second generation alkylphosphocholine (APC). Proc Am Assoc Cancer Res 33:416
4. Bergmann J, Junghahn I, Brachwitz H, Langen P (1994) Multiple effects of antitumor alkyl-lysophospholipid analogs on the cytosolic Ca^{2+} concentration in a normal and a breast cancer cell line. Anticancer Res 14:1549
5. Berkovic D, Grunwald U, Menzel W, Unger C, Hiddemann W, Fleer EAM (1995) Effects of hexadecylphosphocholine on

membrane phospholipid metabolism in human tumour cells. Eur J Cancer 31A:2080
6. Berkovic D, Bensch M, Bertram J, Wille T, Haase D, Binder C, Fleer EAM (2001) Effects of hexadecylphosphocholine on thrombocytopoiesis. Eur J Cancer 37:503
7. Brandes AA, Palmisano V, Pasetto LM, Basso U, Monfardini S (2001) High-dose chemotherapy with bone marrow rescue for high-grade gliomas in adults. Cancer Invest 19:41
8. Clive S, Gardiner J, Leonard RC (1999) Miltefosine as a topical treatment for cutaneous metastases in breast carcinoma. Cancer Chemother Pharmacol [Suppl] 44:S29
9. Davis FG, Freels S, Grutsch J, Barlas S, Brem S (1998) Survival rates in patients with primary malignant brain tumors stratified by patient age and tumor histological type: analysis based on surveillance, epidemiology and end results (SEER) data, 1973–1991. J Neurosurg 88:1
10. Diomede L, Piovani B, Re F, Principe P, Colotta F, Modest EJ, Salmona M (1994) The induction of apoptosis is a common feature of the cytotoxic action of ether-linked glycerophospholipids in human leukemic cells. Int J Cancer 57:645
11. Eibl H, Kaufmann-Kolle P (1995) Medical application of synthetic phospholipids as liposomes and drugs. J Liposome Res 5:131
12. Eibl H, Unger C (1990) Hexadecylphosphocholine, a new and selective antitumor drug. Cancer Treat Rev 17:233
13. Erdlenbruch B, Jendrossek V, Marx M, Hunold A, Eibl H, Lakomek M (1998) Antitumor effects of erucylphosphocholine on brain tumor cells in vitro and in vivo. Anticancer Res 18:2551
14. Erdlenbruch B, Jendrossek V, Gerriets A, Vetterlein F, Eibl H, Lakomek M (1999) Erucylphosphocholine: pharmacokinetics, biodistribution, CNS accumulation and toxicity in the rat after intravenous application. Cancer Chemother Pharmacol 44:484
15. Ergezinger K, Vehmeyer K, Unger C (1999) Stimulation of human hematopoietic progenitor cells by the alkylphosphocholines hexadecylphosphocholine and hexadecyl-N,N,N-trimethyl-hexanolamine. Anticancer Res 19:3213
16. Henke J, Engelmann J, Kutscher B, Nssner G, Engel J, Voegli R, Leibfritz D (1999) Changes of intracellular calcium, fatty acids and phospholipids during miltefosine-induced apoptosis monitored by fluorescence and 13C NMR spectroscopy. Anticancer Res 19:4027
17. Hofer S, Herrmann R (2001) Chemotherapy for malignant brain tumors of astrocytic and oligodendroglial lineage. J Cancer Res Clin Oncol 127:91
18. Jendrossek V, Erdlenbruch B, Hunold A, Kugler W, Eibl H, Lakomek M (1999) Erucylphosphocholine, a novel antineoplastic ether lipid, blocks growth and induces apoptosis in brain tumor cell lines in vitro. Int J Oncol 14:15
19. Jendrossek V, Kugler W, Erdlenbruch B, Eibl H, Lang F, Lakomek M (2001) Erucylphosphocholine-induced apoptosis in chemoresistant glioblastoma cell lines: involvement of caspase activation and mitochondrial alterations. Anticancer Res 21:3389–3396
20. Jorg B, Kerl H, Thiers BH, Bröcker EB, Burg G (1994) Therapeutic approaches in cutaneous lymphoma. Dermatol Clin 12:433
21. Konstantinov SM, Berger MR (1999) Human urinary bladder carcinoma cell lines respond to treatment with alkylphosphocholines. Cancer Lett 144:153
22. Konstantinov SM, Eibl H, Berger MR (1998) Alkylphosphocholines induce apoptosis in HL-60 and U937 leukemic cell lines. Cancer Chemother Pharmacol 41:210
23. Konstantinov SM, Eibl H, Berger MR (1999) BCR-ABL influences the antileukaemic efficacy of alkylphosphocholines. Br J Haematol 107:365
24. Kötting J, Berger MR, Unger C, Eibl H (1992) Alkylphosphocholines: influence of structural variation on biodistribution at antineoplastically active concentrations. Cancer Chemother Pharmacol 30:105
25. Lohmeyer M, Workman P (1993) The role of intracellular free calcium mobilization in the mechanism of action of antitumour ether lipids SRI 62-834 and Et18-OMe. Biochem Pharmacol 45:77
26. Lucas L, Hernandez-Alcoceba R, Penalva V, Lacal JC (2001) Modulation of phospholipase D by hexadecylphosphorylcholine: a putative novel mechanism for its antitumoral activity. Oncogene 20:1110
27. Magistrelli A, Villa P, Benfenati E, Modest EJ, Salmona M, Tacconi MT (1995) Fate of 1-O-octadecyl-2-O-methyl-rac-glycero-3-phosphocholine (ET18-OME) in malignant cells, normal cells, and isolated and perfused rat liver. Drug Metab Dispos 23:113
28. Mollinedo F, Fernandez-Luna JL, Gajate C, Martin-Martin B, Benito A, Martinez-Dalmau R, Modolell M (1997) Selective induction of apoptosis in cancer cells by the ether lipid ET18-OCH3 (Edelfosine): molecular structure requirements, cellular uptake, and protection by Bcl-2 and Bcl-X(L). Cancer Res 57:1320
29. Nicoletti I, Migliorati G, Pagliacci MC, Grignani F, Riccardi C (1991) A rapid and simple method for measuring thymocyte apoptosis by propidium iodide staining and flow cytometry. J Immunol Methods 139:271
30. Noseda A, Godwin PL, Modest EJ (1988) Effects of antineoplastic ether lipids on model membranes and biological membranes. Biochim Biophys Acta 945:92
31. Ries UJ, Fleer EAM, Breiser A, Unger C, Stekar J, Fenneberg K, Eibl H (1993) In vitro and in vivo antitumoral activity of alkylphosphonates. Eur J Cancer 29A:96
32. Ruiter GA, Verheij M, Zerp SF, van Blitterswijk WJ (2001) Alkyl-lysophospholipids as anticancer agents and enhancers of radiation-induced apoptosis. Int J Oncol Biol Phys 49:415
33. Runge MH, Andreesen R, Pfleiderer A, Munder PG (1980) Destruction of human solid tumors by alkyllysophospholipids. J Natl Cancer Inst 64:1301
34. Sobottka SB, Berger MR, Eibl H (1993) Structure-activity relationships of four anti-cancer alkylphosphocholine derivates in vitro and in vivo. Int J Cancer 53:418
35. Überall F, Oberhuber H, Maly K, Zaknun J, Demuth L, Grunicke HH (1991) Hexadecylphosphocholine inhibits inositol phosphate formation and protein kinase C activity. Cancer Res 51:807
36. Wang YZ, Chang YB, Xing C, Fu D (1998) The interference effects of hexadecylphosphocholine on proliferation and membrane phospholipid metabolism in human myeloid leukemia cell lines. Int J Tissue React 20:101
37. Wieder T, Orfanos CE, Geilen CC (1998) Induction of ceramide-mediated apoptosis by the anticancer phospholipid analog, hexadecylphosphocholine. J Biol Chem 273:11025