Cancer Chemother Pharmacol (2005) 56: 261–270
DOI 10.1007/s00280-004-0935-6

ORIGINAL ARTICLE

**Panagiota Papazafiri · Nicolaos Avlonitis
Panagiotis Angelou · Theodora Calogeropoulou
Maria Koufaki · Efi Scoulica · Irene Fragiadaki**

Structure-activity relationships of antineoplastic ring-substituted ether phospholipid derivatives

Received: 23 June 2004 / Accepted: 7 October 2004 / Published online: 26 April 2005

Abstract *Purpose*: Previous studies have shown that alkylphosphocholines (APCs) exhibit strong antineoplastic activity against various tumour cell lines in vitro and in several animal models. The current study was designed to investigate the influence of cycloalkane rings on the antiproliferative activity of APCs against a panel of eight human and animal cell lines (PC3, MCF7, A431, Hela, PC12, U937, K562, CHO). Specifically, we explored the effect of the presence of 4-alkylidenecyclohexyl and cycloalkylidene groups in alkoxyethyl and alkoxyphosphodiester ether lipids, respectively. In addition, the haemolytic activity of the new ring-substituted ether phospholipids (EP) was evaluated. *Methods*: Cells were exposed to various concentrations of the compounds for 72 h. The cytotoxicity was determined with the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] dye reduction assay. Similarly, red blood cells were distributed in 96-well microplates and treated with the test compounds at concentrations ranging from 100 to 6.25 μ*M* for 1 h. After centrifugation, the absorbance of the supernatants was measured at 550 nm. *Results*: The majority of the compounds tested exhibited significant cytotoxic activity which depended on both the ring size and position with respect to the phosphate moiety, as well as the head group. Among the cycloalkylidene series the 11-adamantylideneundecyl-substituted N-methylmorpholino EP 13 was the most potent and exhibited broad-spectrum anticancer activity comparable to or superior to that of hexadecylphosphocholine (HePC). All the adamantylidene-substituted EPs were nonhaemolytic (concentration that exhibits 50% haemolytic activity, HC_{50}, >100 μ*M*). Furthermore, the cyclohexylidene-substituted analogues were more potent against the cell lines tested, with the exception of U937 and K562, than the cyclodecapentylidene-substituted compounds. Hydrogenation of the double bond in the cycloalkylidene-substituted EPs (compounds 14 and 15) resulted in improvement of anticancer activity. Among the 2-(4-alkylidenecyclohexyloxy)ethyl EPs, 2-(4-hexadylidenecyclohexyloxy)ethyl phosphocholine (22) possessed the highest broad-spectrum cytotoxic activity than all the other analogues of this series and was nonhaemolytic (HC_{50} >100 μ*M*). In general, the 2-(4-alkylidenecyclohexyloxy)ethyl-substituted EPs were more active against the more resistant cell lines U937, K562 and CHO than HePC. *Conclusions*: The presence of cycloalkane rings in the lipid portion of APCs reduces haemolytic effects compared to HePC and in several analogues results in improved antineoplastic activity.

Keywords Anticancer · Ether phospholipids · HePC · Haemolysis

P. Papazafiri
Department of Animal and Human Physiology,
School of Biology, University of Athens, Panepistimiopolis,
15784 Athens, Greece

N. Avlonitis · P. Angelou · T. Calogeropoulou (✉) · M. Koufaki
Institute of Organic and Pharmaceutical Chemistry,
National Hellenic Research Foundation,
48 Vassileos Constantinou Avenue, 11635 Athens, Greece
E-mail: tcalog@eie.gr
Tel.: +30-210-7273833
Fax: +30-210-7273831

E. Scoulica · I. Fragiadaki
Department of Clinical Bacteriology, Parasitology,
Zoonoses and Geographical Medicine,
School of Medicine, University of Crete,
76309 Heraklion, Greece

Introduction

Alkyllysophospholipid analogues (ALPs) constitute a promising new class of chemotherapeutics for the treatment of cancer and parasitic infections [4, 7, 8]. The cytotoxic and cytostatic activity of ALPs is remarkably selective for cancer cells and they stimulate host defence by activating macrophage cytotoxicity [15]. Whereas the majority of the conventional anticancer drugs may cause severe side effects due to bone marrow suppression, ALPs are known to exert minimal haematological

toxicity [24, 27]. Additionally, normal resting vascular endothelial cells are not affected by ALPs [31]. This selective antitumour effect is not limited to human cell lines but has also been observed in primary tumour cell cultures from cancer patients. Edelfosine (1, ET-18-OCH_3,1-*O*-octadecyl-2-*O*-methyl-*rac*-glycero-3-phosphocholine) is the prototype of antineoplastic ether phospholipids (EPs) and served as a lead drug for the synthesis of BM 41.440 (ilmofosine), SRI 62-834 and others.

Extensive structure–activity relationship studies on a variety of ALPs have shown that a long alkyl chain and a phosphocholine moiety may represent the minimal structural requirements for sufficient antineoplastic effects of ether lipid analogues [37–39]. This finding led to the synthesis of the alkylphosphocholines (APCs) which exhibit strong antineoplastic activity against various tumour cell lines in vitro [12, 23, 24, 33] and in several animal models [17, 34]. Among the alkyl chain homologs, hexadecylphosphocholine (2, HePC, miltefosine) has therapeutically useful antitumour activity and was approved in Germany in 1992 as a drug for the topical treatment of metastasized mammary carcinoma. Apart from single-agent therapy, APC analogues are potential partners in combination chemotherapy due to lack of bone marrow toxicity and the different mode of action. A synergistic effect on tumour response of HePC combined with the DNA-alkylating agent cyclophosphamide and cisplatin has been shown in chemically induced solid tumours in rats [35, 36]. Additionally, combination experiments with ET-18-OMe, or HePC and radiation, have revealed an enhancement of radiation-induced apoptosis in leukaemic tumour cell lines [5, 31, 32].

However, the use of APCs as anticancer drugs has been limited by their untoward side effects. Specifically, oral administration of HePC results in severe gastrointestinal toxicity, in particular nausea, anorexia, vomiting and diarrhoea [30, 39]. A structural analogue of HePC, perifosine (3, D-21266) has been synthesized and exhibits a better systemic therapeutic index than HePC (Fig. 1). However, a phase I trial has revealed that perifosine has a similar toxicity profile to the parent compound HePC [9]. In addition, intravenous administration of many of the first generation APCs such as HePC is not possible since they induce severe haemolysis as well as necrosis and thrombophlebitis at the injection site [25]. In liposomal formulations, HePC produces no haemolytic effects or local toxicity but is eliminated rapidly from the circulation [10, 21, 22]. Erucylphosphocholine (4, ErPC) is the first intravenously applicable APC which shows strong antineoplastic activity against autochthonous methylnitrosourea-induced mammary carcinomas in rats and brain tumour cell lines in vitro [11, 18, 19]. The ErPC structural analogues docosenyl-(*cis*-10,11)-phosphocholine, tricosenyl-(*cis*-12,13)-phosphocholine, heneicosenyl-(*cis*-12,13)-phosphocholine and erucyl-*N,N,N*-trimethylpropanolaminophosphate reduce cell growth and viability of rat and human astrocytoma/glioblastoma cell lines and show improved antineoplastic activity when compared to HePC while lacking haemolytic activity [19].

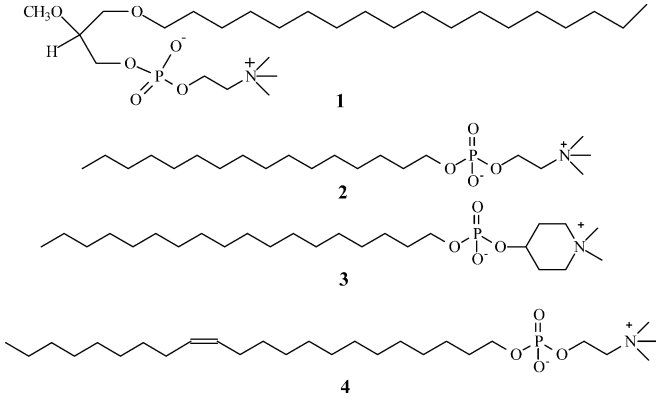


Fig. 1 Structures of edelfosine, HePC, perifosine and ErPC

Although no consensus has emerged regarding the mode of action of these drugs in cancer cells, several likely intracellular targets related to signal transduction and lipid biosynthesis have been identified. Recent data suggest that modulation of cell surface receptors, inositol metabolism, phospholipases, protein kinase C, and other mitogenic pathways may all be involved, thereby inducing differentiation of some cell types and/or apoptosis in others [1, 2, 6, 13, 14, 16, 20, 28].

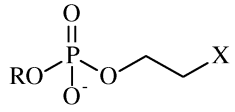
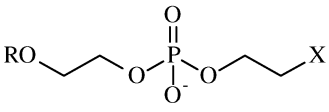
As an ongoing effort aimed at exploring the structural and stereochemical requirements of amphipathic ether lipids for optimal cytotoxicity, immunomodulation and/or antiparasitic activity [3, 26], we studied the effects of cycloalkane rings on the antineoplastic activity of APCs. Specifically, we explored the effect of the presence of 4-alkylidenecyclohexyl and cycloalkylidene groups in alkoxyethyl and alkoxyphosphodiester ether lipids, respectively. These can be envisaged as resulting from the deletion of the C2 or C1-C2 groups from the ALP structure. These compounds have already been evaluated for their activity against *Leishmania* parasites and are severalfold more potent than HePC [3] which was registered for the oral treatment of visceral leishmaniasis in India in March 2002 [8]. Thus, it was of interest to examine whether the anticancer activity of these compounds paralleled their antileishmanial properties.

Materials and methods

Compounds

The structures of the ring-substituted EPs under investigation are presented in Table 1. They can be grouped according to the structure of the lipid portion into cycloalkylidenealkyl-substituted phospholipids 5–13 and 4-alkylidenecyclohexyloxyethyl-substituted phospholipids 16–24. Compounds 14, 15 and 25 are the hydrogenated analogues of EPs 5, 11 and 24, respectively. The compounds were synthesized as previously described [3] or in an analogous manner.

Table 1 Structures of ring-substituted ether phospholipids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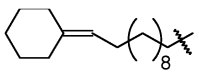
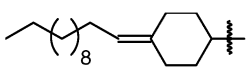
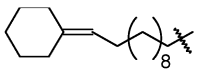
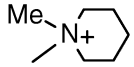
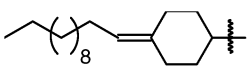
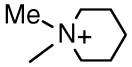
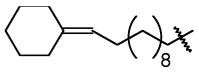
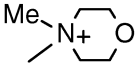
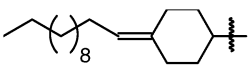
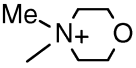
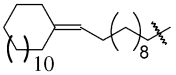
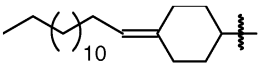
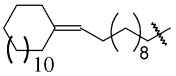
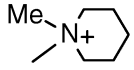
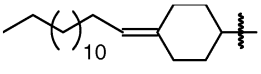
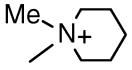
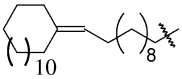
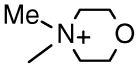
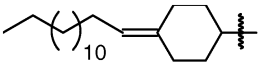
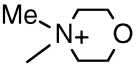
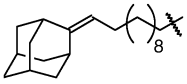
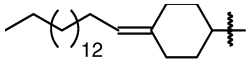
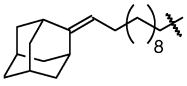
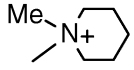
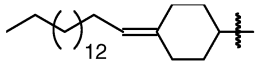
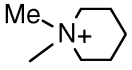
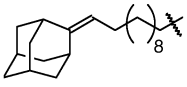
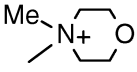
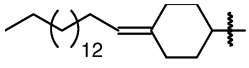
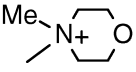
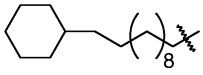
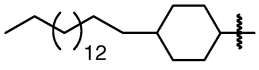
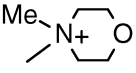
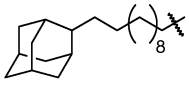
5		$N^+(CH_3)_3$	**16**		$N^+(CH_3)_3$
6			**17**		
7			**18**		
8		$N^+(CH_3)_3$	**19**		$N^+(CH_3)_3$
9			**20**		
10			**21**		
11		$N^+(CH_3)_3$	**22**		$N^+(CH_3)_3$
12			**23**		
13			**24**		
14		$N^+(CH_3)_3$	**25**		
15		$N^+(CH_3)_3$			

Cells

For the assessment of the antitumour activity of the compounds, we used the following panel of cell lines: U937 human promonocytic leukaemia cell line, K562 human chronic myelogenous leukaemia cell line, CHO, from ovary biopsy (Chinese hamster), HeLa derived from human cervix adenocarcinoma (from ECACC), MCF7 derived from a human breast adenocarcinoma, PC3 derived from a human prostate adenocarcinoma, A431 derived from human epidermoid carcinoma (donated by Dr. D. Kletsas), and PC12 phaeochromocytoma (rat adrenal) (donated by Dr. E. Clementi).

Cell culture

Cells were grown as monolayer cultures in Dulbecco's modified Eagle's medium (containing 4.5 g glucose/l) supplemented with 10% fetal bovine serum, 2 m*M*

L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin) at 37°C in an incubator with humidified atmosphere containing 5% CO_2. Cells were passaged by trypsinization once or twice a week to keep them in log phase. K562 and U937 cells were grown as suspension cultures in RPMI 1640 medium under the same conditions.

Cytotoxicity determination

Cells were seeded into 96-well plates (100 μl/well at a density of 1×10^5 cells/ml) and exposed to various concentrations of the compounds for 72 h. The cytotoxicity was determined with the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] dye reduction assay as described by Mosmann [29] with minor modifications. Briefly, after incubation with the test compounds, MTT solution (5 mg/ml in PBS) was added (20 μl/well). Plates were further incubated for 4 h at 37°C and the formazan crystals formed were dissolved by adding 100 μl/well of 0.1 *N* HCl in 2-propanol. Absorption was measured by an enzyme-linked immunosorbent assay (ELISA) reader at 545 nm, with reference filter at 690 nm. For each concentration at least nine wells were used from three separate experiments. RPMI 1640 or DMEM (100 μl) supplemented with the same amount of MTT solution and solvent was used as blank solution. The data obtained are presented as IC_{50} ± SEM (μ*M*), which is the concentration of the compound causing a reduction in the number of viable cells to 50% of the untreated control cells.

Trypan blue exclusion

Following drug treatments, the medium in each plate was collected into a centrifuge tube. Cells attached to wells were removed by trypsinization and pooled with the medium. Cells were centrifuged at 250 *g* for 5 min, and the pellet was resuspended and incubated in PBS with 0.1% trypan blue for at least 5 min. Viability was determined as the percentage of viable cells over the total number of cells. A total of 200–400 cells were counted by two investigators without knowledge of the respective treatments, and the mean was used for statistical analysis.

DNA laddering

Apoptosis was determined by genomic DNA fragmentation assessed by agarose gel electrophoresis. Treated or untreated A431 cells were rinsed once with PBS without Ca^{2+} and Mg^{2+} and trypsinized. The cell pellet was redissolved in suspension buffer (100 m*M* Tris-Cl, pH 8.0, 200 m*M* NaCl, and 10 m*M* EDTA) and lysed in digestion buffer containing 100 m*M* Tris-Cl, pH 8.0, 200 m*M* NaCl, 10 m*M* EDTA, 0.4% SDS, and 100 μg proteinase K per sample. After incubation at 55°C overnight, DNA was extracted with phenol/chloroform (1:1) and after centrifugation, the upper aqueous layer was transferred to new tubes and extracted with an equal volume of chloroform. Samples were then centrifuged, the aqueous layer was transferred to new tubes and incubated in the presence of RNAse (20 μg/ml) for 30 min at 37°C. Finally, DNA was precipitated with 100% ethanol, then dissolved in distilled water, and quantified by measuring absorbance at 260 nm. Equivalent amounts of DNA (10 μg) were analysed by gel electrophoresis in a 1% agarose gel and stained with ethidium bromide.

Haemolysis of red blood cells

EDTA-preserved peripheral blood from healthy volunteers was centrifuged to remove the serum, and the red blood cells were washed thrice in PBS. After the final wash, cells were distributed in 96-well microplates (100 μl/well) and an equal volume of each compound concentration was added. The test compounds were diluted in PBS at concentrations ranging from 100 to 6.25 μ*M*. After incubation at 37°C for 1 h, red cells were centrifuged at 800 *g* for 10 min. Absorbance of the supernatants was measured at 550 nm with the reference filter at 625 nm. The percentage haemolytic activity of each drug at different concentrations was estimated as $(A-A_0/A_{max}-A_0)\times100$ where A_0 is the background haemolysis obtained by incubation with PBS and A_{max} is the 100% haemolysis achieved after incubation in distilled water.

Results

The results of the MTT assay after 72 h exposure are presented in Tables 2 and 3. IC_{50} values were calculated for each test compound for HeLa, U937, K562, A431, PC3, CHO, MCF7 and PC12 cells. The majority of the compounds tested exhibited significant antineoplastic activity, although this effect varied among the cell types. K562 and CHO cells were the most resistant while PC3 and MCF7 were the most sensitive cells. Resistance of K562 cells against APCs has already described been by Konstantinov et al. [24]. The first series of compounds (5–13) have cyclohexylidene, cyclodecapentylidene or adamantylidene moieties linked to the polar head group by an oligomethylene bridge of 11 carbons. Thus, we examined the effects on activity of the ring size in combination with changes in the polar portion while the chain length was maintained constant (Table 2). More specifically, the cyclohexylidene-substituted analogues (5–7) were more potent against the cell lines tested, with the exception of U937 and K562 cells, than the cyclodecapentylidene-substituted compounds (8–10). Among the head group congeners of compounds 5–7 the trimethylammonio-substituted 5 was the most potent against

Table 2 IC_{50} values (μ*M*) after 72 h of exposure to the compounds. IC_{50} is the concentration of the compound where $100\times(A_0-A)/A_0=50$, where A is the optical density of the wells after 72 h of exposure to test compound and A_0 is the optical density of the control wells. The data presented are means ± SEM of three separate experiments

Compound	Cell line							
	PC3	MCF7	A431	HeLa	PC12	U937	K562	CHO
5	12.41 ± 2.2	34.07 ± 2.45	35.47 ± 3.3	40.3 ± 3.74	76.06 ± 3.46	84.76 ± 3.74	> 100.0	95.18 ± 4.24
6	17.25 ± 2	44.28 ± 3.6	39.22 ± 3.56	45.21 ± 3.16	68.33 ± 3.74	89.65 ± 4.24	78.33 ± 3.6	87.31 ± 4
7	19.56 ± 2.64	54.39 ± 3.16	41.68 ± 3	30.42 ± 2.83	74.91 ± 3.3	> 100.0	> 100.0	79.64 ± 3.74
8	56.78 ± 4.88	61.37 ± 4.6	86.41 ± 5.21	72.8 ± 3.75	> 100	62.01 ± 4.63	58.92 ± 8.4	> 100
9	64.37 ± 3.6	52.18 ± 4.83	92.61 ± 6.98	68.48 ± 4.1	84.77 ± 6.1	74.32 ± 5.7	72.63 ± 5.81	> 100
10	53.46 ± 4.39	58.72 ± 6.11	71.35 ± 6.2	68.07 ± 3.74	74.88 ± 5.78	63.46 ± 4.76	61.78 ± 8.28	91.54 ± 8.62
11	42.65 ± 2.3	47.72 ± 3.6	39.22 ± 3.74	44.76 ± 2.8	72.18 ± 3.87	82.15 ± 4.6	65.41 ± 3.46	87.64 ± 3.6
12	60.43 ± 3.6	48.17 ± 3.84	80.2 ± 3.6	85.18 ± 3.6	77.46 ± 4	73.42 ± 3.3	55.45 ± 3.4	90.28 ± 3.87
13	30.5 ± 3.16	24.77 ± 2.6	29.83 ± 3	39.84 ± 3.46	47.85 ± 4	55.0 ± 3.87	49.51 ± 3.74	74.66 ± 3.6
14	8.76 ± 2.2	20.6 ± 3.6	18.45 ± 3.6	27.4 ± 3.71	54.21 ± 3.88	46.71 ± 4.2	98.32 ± 4.6	74.28 ± 3.9
15	11.4 ± 2	19.41 ± 2.45	21.34 ± 3.1	26.85 ± 4	47.91 ± 3.88	38.91 ± 3.84	87.16 ± 6.2	74.3 ± 6.78
HePC	13.76 ± 3.16	17.34 ± 3	24.56 ± 2.45	32.17 ± 3	34.82 ± 3.16	39.71 ± 3.46	55.43 ± 3.6	87.23 ± 4.58

PC3, MCF7, A431 and U937 cells, the N-methylpiperidino 6 against PC12 and K562 cells,and the N-methylmorpholino 7 against HeLa and CHO cells. Concerning the head group SAR of compounds 8–10, the trimethylammonio-substituted 8 was the most potent against K562 cells, the N-methylpiperidino 9 against MCF7 cells, and the N-methylmorpholino 10 against PC3, A431, PC12 and CHO cells. Analogues 9 and 10 were equipotent against HeLa cells while compounds 8 and 10 were equipotent against U937 cells.

Among the adamantylidene-substituted analogues (11–13) the N-methylmorpholino derivative 13 was the most potent against all cell lines tested. Interestingly, compound 13 was the most potent of the cycloalkylidene-substituted EPs against MCF7, A431, PC12, U937, K562 and CHO cells, and more potent than HePC against CHO and K562 cells. In addition, the hydrogenated derivatives of the choline-substituted cyclohexylidene and adamantylidene analogues 5 and 11, compounds 14 and 15, respectively, were synthesized in order to examine the influence of the double bond on anticancer activity. This transformation resulted in an improvement in activity from 1.5-fold to 3.7-fold against the majority of cell lines tested (Table 2). Analogues 14 and 15 were equipotent against MCF7, HeLa and CHO cells, while 14 was more potent than 15 against PC3 and A431 cells, and 15 was more potent than 14 against PC12, U937 and K562 cells. Futhermore, 14 and 15 exhibited higher activity than HePC against PC3, A431, HeLa and CHO cells.

The second series comprised 2-(4-alkylidenecyclohexyloxy)ethyl-substituted phospholipids 16–24 (Table 1), and their cytotoxic activities are shown in Table 3. Among the 2-(4-dodecylidenecyclohexyloxy)ethyl congeners, compounds 16–18, the N-methylpiperidino derivative 17 was the least active while, the N-methylmorpholino analogue 18 exhibited significant cytotoxicity against all cell lines tested, being more potent than HePC against HeLa, K562 and CHO cells and equipotent against MCF7, A431 and U937 cells.

Introduction of a tetradecylidene alkyl chain rendered the choline-substituted derivative 19 the most potent of the head group congeners. In addition, the activity was improved with respect to analogue 16 in PC3, MCF7 and HeLa cells, remains unchanged in PC12 cells and decreased in A431 cells.

Table 3 IC_{50} values (μ*M*) after 72 h of exposure to the compounds. IC_{50} is the concentration of the compound where $100\times(A_0-A)/A_0=50$, where A is the optical density of the wells after 72 h of exposure to test compound and A_0 is the optical density of the control wells. The data presented are means ± SEM of three separate experiments (*ND* not determined)

Compound	Cell line							
	PC3	MCF7	A431	HeLa	PC12	U937	K562	CHO
16	20.9 ± 3.16	24.05 ± 2.83	27.11 ± 2.45	31.93 ± 2.64	40.16 ± 3.46	ND	ND	ND
17	27.49 ± 3.3	34.21 ± 3	51.77 ± 3.74	65.5 ± 3.46	70.36 ± 4	ND	ND	ND
18	18.65 ± 2.4	15.23 ± 3.16	28.34 ± 4.4	13.38 ± 3.61	58.92 ± 7.2	42.37 ± 4.6	26.3 ± 3.65	72.45 ± 4.5
19	14.71 ± 2	20.35 ± 2.65	34.71 ± 3.73	25.37 ± 2.83	40.27 ± 3.46	ND	ND	ND
20	75.39 ± 2.45	79.6 ± 3.16	65.4 ± 3.16	90.4 ± 3.3	69.7 ± 3.74	ND	ND	ND
21	63.4 ± 3	88.8 ± 3.16	57.3 ± 3.46	49.58 ± 3.74	75.41 ± 3.6	ND	ND	ND
22	22.67 ± 3.41	17.81 ± 4.2	27.95 ± 5.7	21.54 ± 3.46	38.56 ± 8.56	28.93 ± 2.8	29.23 ± 3.76	51.10 ± 4.73
23	28.23 ± 3.1	23.54 ± 3.25	34.3 ± 3.41	33.15 ± 2.3	51.12 ± 3.75	35.14 ± 3.16	31.23 ± 3.8	60.34 ± 4.68
24	90.16 ± 4.24	85.14 ± 4.15	80.22 ± 3.75	> 100.0	> 100.0	ND	ND	ND
25	65.2 ± 2.83	69.7 ± 3.87	> 100.0	80.24 ± 4.12	> 100.0	ND	ND	ND
HePC	13.76 ± 3.16	17.34 ± 3	24.56 ± 2.45	32.17 ± 3	34.82 ± 3.16	39.71 ± 3.46	55.43 ± 3.6	87.23 ± 4.58

Among the 2-(4-hexadecylidenecyclohexyloxy)ethyl series, the trimethylammonio derivative 22 was the most potent against all cell lines tested, being more potent than HePC against HeLa, U937, K562 and CHO cells, equipotent to HePC against MCF-7 cells, and slightly less potent against A431 and PC12 cells. Hydrogenation of the double bond in analogue 24, compound 25, resulted in improvement in activity against PC3, MCF-7 and HeLa cells, but a decrease against A431. As a general remark, the 2-(4-alkylidenecyclohexyloxy)ethyl-substituted EPs were more active against the more resistant cell lines U937, K562 and CHO than HePC.

Cell viability was also determined by the trypan blue exclusion test for the most active of the new compounds and for three sensitive and one resistant cell line (PC3, MCF7, A431 and K562, respectively; Table 4). Evaluation of cell viability by the MTT test and by trypan blue gave similar results (Table 4). In addition, concentration-effect curves of the most potent compounds (6, 13, 14, 18, 22) revealed a clear cytotoxic effect of these compounds (Fig. 2).

The electrophoretic separation of DNA purified from A431 cells exposed to selected compounds that exhibited potent cytotoxic activity showed a DNA fragmentation profile typical of programmed cell death (Fig. 3). Compounds 13 and 22 induced prominent ladder formation similar to that induced by HePC, whereas a reduced apoptotic effect was observed in the presence of compound 18 (lane 4, Fig. 3). On the contrary, when A431 cells were incubated in the presence of compound 6 (at the same concentration), no fragmentation was detected, indicating direct cell necrosis.

Since haemolysis is a problem associated with APCs which prevents their use in injectable form, it is important to study the haemolytic properties of the new compounds. Thus, the haemolytic activity of several of the new analogues was evaluated against red blood cells (Fig. 4, Table 5). The results are expressed as the concentration that exhibited 50% haemolytic activity (HC_{50}) and are shown in Table 5. All the compounds tested exhibited lower haemolytic activity than HePC. Specifically, from the first series, analogues 6 and 11–13 possessed HC_{50} values $>100\ \mu M$, while compound 5 had an HC_{50} value of $68.31 \pm 3.39\ \mu M$. The hydrogenated derivatives 14 and 15 also possessed HC_{50} values $>100\ \mu M$. Among the second series, only the 2-(4-dodedecylidenecyclohexyloxy)ethyl-substituted choline derivative 16 and the 2-(4-dodedecylidenecyclohexyloxy)ethyl-substituted N-methylpiperidino derivative 17 exhibited haemolytic activity with HC_{50} values of 40.65 ± 6.65 and $58.75 \pm 2.23\ \mu M$, respectively. Analogues 18–20 and 22–24 all possessed HC_{50} values $>100\ \mu M$.

Table 4 Cell viability after 72 h of exposure to the compounds. Viability was determined in terms of trypan blue exclusion as the percent of viable cells over the total number of cells

Compound	Cell line			
	PC3	MCF7	A431	K562
6	24.3 ± 4.2	51.4 ± 5.8	43.5 ± 3.9	>100
13	37.4 ± 6.7	27.2 ± 4.1	32.2 ± 4.5	58.9 ± 7.2
14	13.8 ± 4.6	23.7 ± 2.1	23.6 ± 2.7	>100
15	14.6 ± 4.1	24.6 ± 3.7	27.1 ± 2.6	>100
18	21.9 ± 3.5	19.8 ± 2.2	32.6 ± 3.7	39.2 ± 4.7
22	29.5 ± 5.3	21.4 ± 3.4	35.9 ± 4.0	35.4 ± 4.1
23	32.1 ± 4.8	27.4 ± 4.5	37.8 ± 4.4	41.7 ± 3.2
HePC	17.2 ± 3.7	21.2 ± 3.2	29.1 ± 3.4	68.5 ± 8.2

Discussion

We describe structure-activity relationships of ring-substituted EPs against a panel of eight cancer cell lines. Structural modifications of the lipid portion as well as modification of the polar phosphocholine group significantly alter the antineoplastic activity of the new EPs. In the majority of the cell lines investigated, a significant antineoplastic activity was seen which depended both on the ring size and position with respect to the phosphate moiety as well as the head group. Our findings from the trypan blue exclusion assay suggest a putative cytotoxic effect rather than a cytostatic one for the most active of the EPs used in the present study. This corroborates with the dose-response curves obtained by the MTT survival assay (Fig. 2). Notably, all the compounds investigated caused cell death in a concentration-dependent manner.

The extent of the cytotoxic effects of the new analogues depended on the tumour cell line investigated. Human tumour cell lines, with the exception of K562, were more sensitive, which is consistent with the activity of HePC. With regard to the effect of ring size on anticancer activity, among the choline congeners of 11-cycloalkylidene-undecyl-substituted EPs (5, 8, 11), a cyclohexane ring was preferred for highest activity against the sensitive cell lines (PC3, MCF7, A431 and HeLa), while a cyclodecapentyl moiety was preferred for the resistant cell lines K562 and U937, and an adamantane ring for the PC12 and CHO cell lines. Among the N-methylpiperidino congeners (6, 9, 12), the same trend was found for the sensitive cell lines, while for the resistant ones there was no clear general preference for ring size. Finally, for the N-methylmorpholino derivatives a cyclohexane ring conferred the highest activity against the sensitive cell lines PC3 and HeLa, while the adamantane moiety was preferred for cytotoxicity against all the remaining cell lines under study.

An analogous comparison of the effect of the alkyl chain on anticancer activity within the head group congeners of 2(4-alkylidenecyclohexyloxy)ethyl-substituted EPs led to the following observations (Table 3). Among the choline congeners (16, 19, 22) there was no clear trend for preferred chain length for improved cytotoxicity. Conversely, for the N-methylpiperidino-substituted EPs (17, 20, 23) a 16-carbon chain rendered the respective compounds more active against all cell

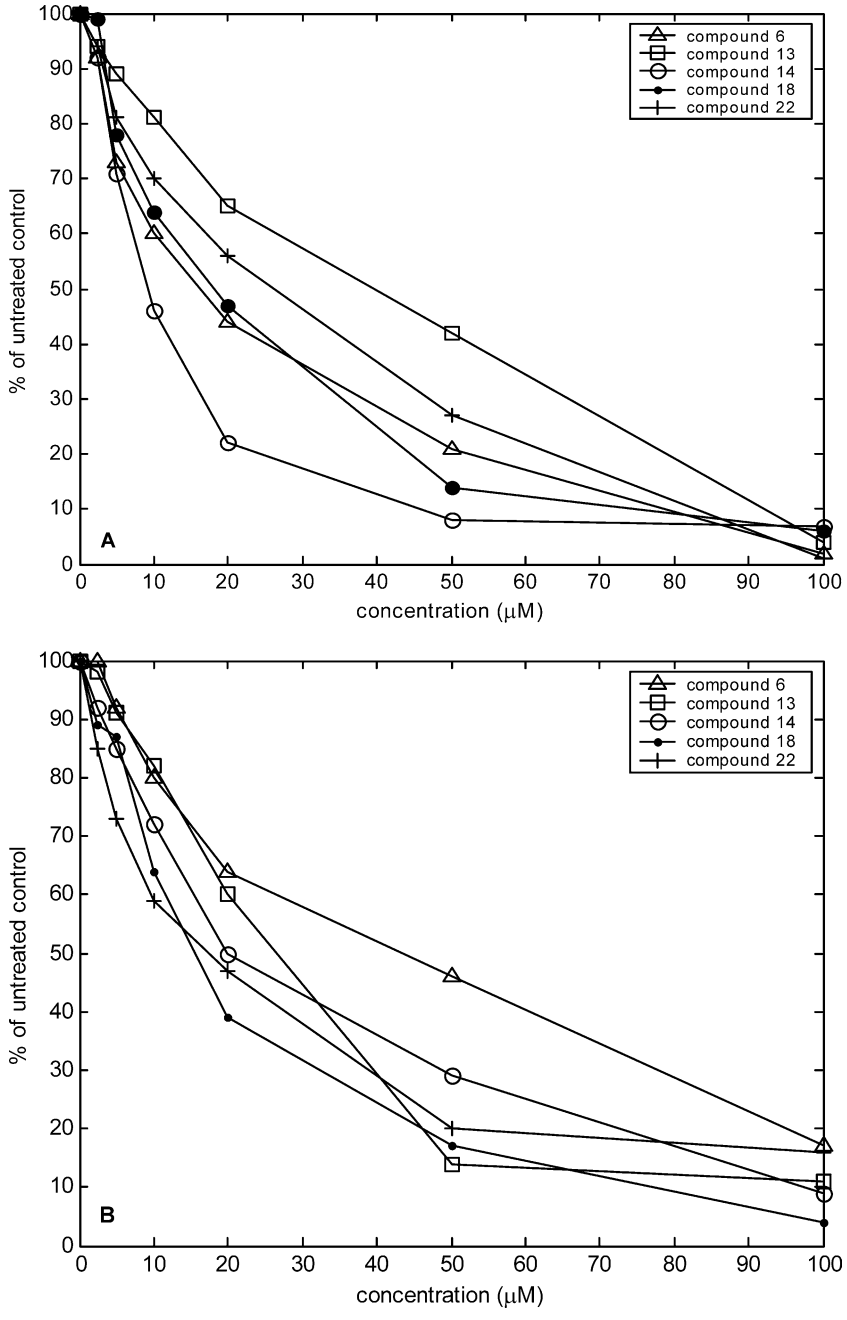


Fig. 2 Typical concentration-effect curves of selected compounds in PC3 cells (**A**) and MCF7 cells (**B**) as measured by the MTT assay after 72 h of exposure (SEM, omitted for clarity, was in all cases ≤ 10% of the mean)

lines tested. Surprisingly, for the N-methylmorpholino derivatives (18, 21, 24) a dodecyl alkyl chain was preferred. This is in contrast to the observation that for APCs cytotoxicity increases with increasing chain length. In any case, an increase in chain length may alter the physical characteristics of the compounds influencing their uptake and distribution.

Concerning their haemolytic activity, the majority of the new compounds possessed HC_{50} values higher than 100 μ*M*. Comparing the percent haemolysis at the highest concentration tested (100 μ*M*) (Table 5), there were differences which depended both on the lipid portion and the head group, but there was no clear trend as to which combination of structural features was the optimum. More importantly, the most cytotoxic of the new EPs in the present study exhibited low haemolytic activity with an HC_{50} value higher than 100 μ*M*. In particular, compounds 13, 14 and 15 from the first series at 100 μ*M* induced 38.80%, 42.80% and 37.80% haemolysis, respectively. Furthermore, analogues 18, 19, 22 and 23 from the second series at 100 μ*M* induced 37.51%, 26.20%, 8.30% and 7.90% haemolysis, respectively. This indicates that a number of the new compounds could be given intravenously and thus they may show reduced gastrointestinal toxicity and higher plasma concentrations in vivo.

Fig. 3 DNA laddering in A431 cells treated for 72 h with compound 6 (*lane 2*), compound 13 (*lane 3*), compound 18 (*lane 4*), compound 22 (*lane 5*) or HePC (*lane 6*) (*lane 1* untreated control). All compounds were used at a concentration of 20 μ*M*

As one could derive from their lipid-soluble structure, the main target of action of antitumour EPs appears to be the plasma membrane. Despite investment of much effort, the exact mechanism of action has not been fully elucidated, but several observed effects provide clues to the nature of the cytotoxic and cytostatic actions of these drugs which may involve immunological modulation as well as direct effects on signal transduction [33, 34]. More than one mechanism might be responsible for the different sensitivities to these compounds. These might be due to differences in drug uptake, the activity of phospholipid-metabolizing enzymes or the constitutive activity of key signalling molecules such as protein kinase C. Alternatively, different sensitivities might be associated with increased efflux of drugs as by MDR1,MDR2, MRP or LRP (lung-resistant protein) transport proteins. Furthermore, since it has been shown that phospholipid analogues induce apoptosis, p53 function and response to induction of apoptosis may also be responsible for differences in sensitivity.

In order to get an insight into the process by which these compounds exert their cytotoxic effects, a DNA laddering assay using the sensitive cell line A431 was performed for four of the new EPs (compounds 6, 13, 18, 22). Our findings indicate that cytotoxic concentrations were able to induce apoptosis. Remarkably however, compound 6 induced necrosis. Compounds 6 and 13 differ both in the lipid portion and the head group being substituted by a cyclohexylidene group and N-methylpiperidino and adamantylidene moiety and choline, respectively. Compounds 18 and 22 belong to the 2-(4-alkylidenecyclohexyloxy)ethyl series and they bear an N-methylmorpholino-substituted head group and choline, respectively. It is conceivable that the difference in the head group in compound 6 is responsible for the observed necrosis rather than apoptosis in the A431 cell line, but further experiments are required to support this hypothesis. Our findings suggest that apoptosis is involved in the cytotoxic effect of some of the new compounds against the A431 cell line.

In summary, the presence of a cycloalkane ring in the lipid portion of APCs reduces haemolytic effects compared to HePC and in several analogues results in improved antineoplastic activity. In particular, for the cycloalkylidenealkyl-substituted derivatives it is noteworthy that significant cytotoxicity was achieved even though the oligomethylene bridge between the ring and the phosphate moiety is only 11 carbons long. The 11-adamantylidene undecyl-substituted N-methylmorpho-

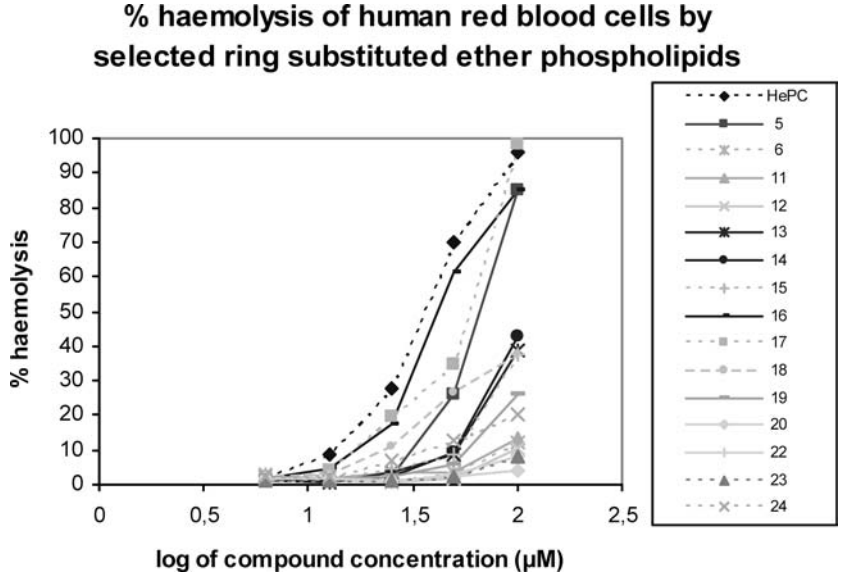


Fig. 4 Haemolytic activities of selected ring-substituted EPs. For the chemical structure of the compounds, refer to Table 1 and Fig. 1

Table 5 Haemolytic activity of selected compounds. Red blood cells were treated with the test compounds diluted in PBS at concentrations ranging from 100 to 6.25 μ*M*. After 1 h, the haemoglobin content was determined by an in vitro assay. The percentage haemolytic activity of each drug at different concentrations was estimated as $(A-A_0/A_{max}-A_0)\times100$, where A_0 is the background haemolysis obtained by incubation with PBS and A_{max} is 100% haemolysis achieved after incubation in distilled water. HC_{50} (μ*M*) is the concentration of the compound where $100\times(A_0-A)/A_0=50$. The data presented are means ± SEM of three separate experiments. For the chemical structure of the compounds, refer to Table 1 and Fig. 1

Compound	HC_{50} (± SEM, μ*M*)	Percent haemolysis at 100 μ*M*
5	68.31 ± 3.39	
6	> 100	12.43
11	> 100	13.20
12	> 100	10.60
13	> 100	38.80
14	> 100	42.80
15	> 100	37.80
16	40.65 ± 6.65	
18	> 100	37.51
19	> 100	26.20
20	> 100	3.80
21	58.75 ± 2.23	
22	> 100	8.30
23	> 100	7.90
24	> 100	20.10
HePC	38.26 ± 2.83	

lino EP 13 possessed the highest broad-spectrum activity of the cycloalkylidene series. The hydrogenated analogue 11-adamantylundecylphosphocholine (15) exhibited broad-spectrum anticancer activity comparable or superior to that of HePC, except against K562 and PC12 cells. Both 13 and 15 were nonhaemolytic (HC_{50} > 100 μ*M*). Among the 2-(4-alkylidenecyclohexyloxy)ethyl EPs, 2-(4-hexadylidenecyclohexyloxy)ethyl phosphocholine (22) possessed the highest broad-spectrum cytotoxic activity than all the other analogues of this series. Interestingly, it was also more potent than HePC against the resistant lines U937, K562 and CHO.

Acknowledgements This work was supported in part by the GSRT program 02PRAXE83. P. Papazafiri acknowledges support from the GSRT program EPAN YB/39.

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