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Antineoplastic activity *in vitro* of free and liposomal alkylphosphocholines

Reinhard Zeisig,[1] Sabine Jungmann,[1] Dieter Arndt,[1] Anneliese Schütt[1] and Eberhard Nissen[2]

[1]Max Delbrück Center for Molecular Medicine, Robert Rössle Straße 10, 0-1115 Berlin-Buch, Germany. Fax: (030) 9494161

[2]Robert Koch Institut des Bundesgesundheitsamtes, Britzer Straße 1–3, 0-1190 Berlin, Germany

We investigated the liposome forming properties of three homologues of alkylphosphocholines: hexadecylphosphocholine (HPC), octadecylphosphocholine (OPC) and eicosanylphosphocholine (EPC). In the presence of cholesterol and dicetylphosphate, alkylphosphocholines form liposomes with slow permeability for entrapped carboxyfluorescein. We studied the direct cytotoxicity of alkylphosphocholine vesicles and their ability to attack MethA sarcoma cells, human skin and muscle fibroblasts (M22, GUS, Moscow), and human mouth epidermoid carcinoma cells (KB, ATCC, CCL 17). All alkylphosphocholines show cytotoxic activity against the investigated cells, the degree of which depends on the number of carbon atoms in the alkyl chain, concentration and incubation time. Whereas the etherlipid liposomes are less toxic to MethA cells than the free compounds, the liposomal alkylphosphocholines are more toxic toward KB and M22 cells than the corresponding free lipids.

Key words: Alkylphospholipid, antineoplastic activity, eicosanylphosphocholine, etherlipid, hexadecylphosphocholine, liposomes, octadecylphosphocholine.

Introduction

Etherlipids – alkyl lysophospholipids and alkylphospholipids – have been found to have a high level of physiological activity[1–4]. Some of these agents are cytotoxic, inhibit tumor growth,[5,6] lead to lysis of blood cells,[7] activate macrophages[8,9] or induce cell differentiation of leukemic cells.[10]

Apart from the well-investigated Et-18-OCH_3,[2,11] interest is now being increasingly focussed on alkylphosphocholines, especially on hexadecylphosphocholine (HPC), which in spite of its simple structure (Figure 1), shows high selective toxicity against a number of leukemic cell lines *in vitro*.[12] Furthermore this etherlipid shows *in vivo* cancerostatic activity against chemically induced mammary carcinoma of the rat,[13,14] and against murine and human mammary carcinoma.[15] Clinical trials on the use of HPC in the therapy of metastatic mammary carcinoma are promising.[16]

Until now, only a few attempts have been made to use etherlipids for the preparation of liposomes.[17–19] Liposomal encapsulation is an accepted possibility for changing the biodistribution and pharmacokinetics of drugs, aimed at increasing the activity of the drug and preventing side effects.[20] Furthermore, the encapsulation of cytostatic drugs in etherlipid liposomes with a cytostatic activity of their own should be a way to combine the therapeutic effects of the etherlipid and drug, and could result in additional or desirable synergistic effects against some tumors.

As a first step towards such experiments we have investigated the liposome-forming properties of HPC and some of its analogs with different alkyl chain lengths (C_{18} and C_{20}).

In a continuation of our initial *in vivo* experiments,[21] we now present data on the cytotoxic properties of these liposomes compared with the free etherlipids *in vitro*.

Materials and methods

Substances

Hexadecanol, octadecanol, dicetylphosphate (Serva, Heidelberg, Germany), eicosanol (Merck, Darmstadt, Germany), dipalmitoylphosphorylglycerol (Sigma, Deisenhofen, Germany) and Triton X-100 (Ferak, Berlin, Germany) were used without further purification. Mitoxantrone (AWD, Dresden, Germany) and daunorubicine (Medexport, USSR) were used as cytostatics.

All solvents were distilled and dried before use.

Correspondence to R Zeisig

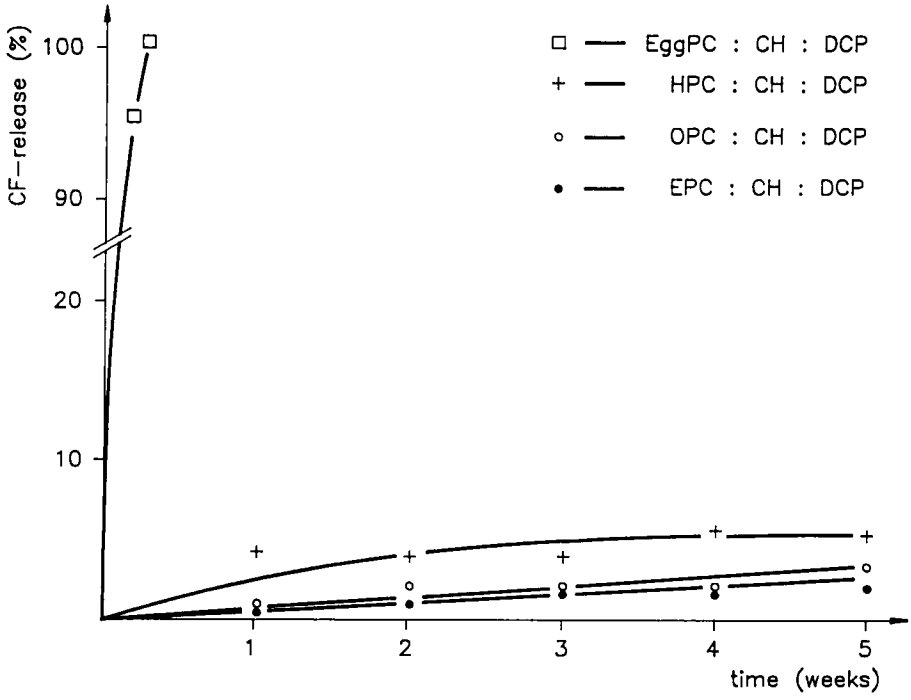


Figure 1. CF release from alkylphosphocholine liposomes. SUV composition was 1:1:0.2 (lipid:CH:DCP). At different times the content of retained CF was determined spectrophotometrically after destroying the liposomes by addition of 1% Triton X-100. For details see Materials and methods.

6(5)-Carboxyfluorescein (CF) (Eastman Kodak, Rochester, NY) was treated with activated charcoal and then separated on Sephadex LH 20.[22] Cholesterol (CH, from Serva) was recrystallized from acetone. [Methyl-^{3}H]thymidine was from Amersham Buchler (Braunschweig, Germany).

Synthesis of alkylphosphocholines

The alkylphosphocholines were prepared by a modified synthesis from the respective intermediates alkyl phosphoric-acid-β-bromethyl-ether[23,24] by hydrolyzation and trimethylamination[25] as follows.

From alcohol, β-bromethyl-phosphorus acid-dichloride and triethylamine was obtained the phosphorus acid-diether-monochloride in tetrahydrofuran (THF). After 4 h the stirring was stopped, the solution filtered and the residue co-distilled with toluene after evaporation of the solvent. The product was dissolved in THF and hydrolyzed by the addition of water and stirred for 5 h. The organic solvent was evaporated in vacuum and the aqueous phase was allowed to stand overnight at 4°C to obtain a weak yellow precipitate. It was used without further purification.

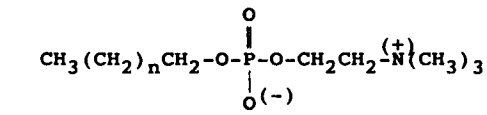
This β-bromoderivative was then dissolved in $CHCl_3$:isopropanol:dimethylformamide (3:5:5, v/v/v). Trimethylamine in water (45%) was added and the solution was stirred at 50°C for 24 h. The solvent was evaporated and the alkylphosphocholine separated by column chromatography using silica gel 60 (230–400 mesh ASTM; Merck) and the solvent system $CHCl_3$:methanol:ammonia (25% in water) 78:30:4.7 (v/v/v). The resulting oily product was precipitated by the addition of acetone.

The structure and purity of the alkylphosphocholine were guaranteed by elemental analysis (for the dihydrates), thin layer chromatography and fast atom bombardment-mass spectroscopy (FAB-MS). All data are in agreement with expectations (Table 1).

Preparation of liposomes

The vesicles were prepared according to Szoka[26] in different combinations (Table 2). The required amounts of etherlipid, cholesterol and, in some cases, dicetylphosphate or dipalmitoyl-phosphatidylglycerol were mixed and dissolved in chloroform:methanol (9:1, v/v). The solvent was removed by rotary evaporation and the resulting lipid film dried for 2 h under reduced pressure.

Table 1. Structure, molecular weight and analytical data for used alkylphosphocholines



	HPC	OPC	EPC
n	14	16	18
M (g/mol)	407.59	435.64	463.67
		FAB-MS	
M^+	408	436	464
Rel. Int.[a]	47.10	23.05	30.23
		Elemental analysis[b]	
H cal:	11.37	11.55	11.94
found:	11.15	11.24	11.72
C cal:	59.27	60.90	62.34
found:	58.98	59.24	61.78
N cal:	3.29	3.08	2.91
found:	3.40	2.94	2.88
P cal:	7.28	6.88	6.43
found:	6.98	6.65	6.40

[a]Intensity M^+ to basic peak (in %).
[b]Calculated for APC × 2 H_2O.

Multilamellar vesicles (MLV) were formed by resuspending the dry lipid film with phosphate buffered saline solution (PBS), 150 mM carboxyfluorescein solution, or a cytostatics buffer solution (1.58 mM mitoxantrone in PBS; 2.73 mM daunorubicine in HEPES) and were shaken overnight.

For preparing small unilamellar vesicles (SUV), the solution was sonicated by a Branson sonifier B15, intermittently for 6 × 4 min, 1:1 pulsed input, with cooling. The suspension was allowed to stand at room temperature for 1 h. After centrifugation at 40 000 *g* for 1 h the liposome suspension was passed over a column (1.5 × 25 cm) of Sephadex G50 medium and eluted with PBS to remove unentrapped carboxy-fluorescein or drug, respectively.

For the determination of CF leakage, the purified CF liposomes were stored at 4°C for 5 weeks. The content of CF was determined by fluorescence spectroscopy weekly and compared with the initial dye content at the starting time.

Reverse-phase evaporation vesicles (REV) were formed by dissolving the lipid film in a mixture of freshly distilled diisopropylether and chloroform (1:1). The aqueous solution of the material, which has to be entrapped, was added to the mixture (ratio of 1:6, organic solvent:aqueous solution) and the suspension was briefly sonicated. Then the organic solvents were removed under reduced pressure. In the case of MLV and REV, unentrapped CF was removed by centrifugation of the liposomes at 30 000 *g* and washed twice with PBS.

For the preparation of liposome–drug complexes with the pH gradient method,[27] empty SUV were made under addition of citric acid, pH 4.0, and a transmembrane gradient was generated by column chromatography (Sephadex G50) with HEPES buffer, pH 7.4. The liposomes were then incubated with mitoxantrone or daunorubicine in PBS. The unentrapped drug was separated from liposomes by gel chromatography (Sephadex G50). The liposomal drug was determined spectrometrically at 485 or 625 nm.

Table 2. Size and encapsulation capacity (*e*) of alkylphosphocholine liposomes of different lipid composition

Liposome type	Lipid composition	Size (nm)	*e* (%)
SUV	HPC:CH	60	
	HPC:CH:DCP	47	0.5
	OPC:CH:DCP	55	1.0
	EPC:CH:DCP	50	0.6
MLV	HPC:CH	580	
	HPC:CH:DCP[a]	500	1.0
	OPC:CH:DCP[a]	437	2.3
	EPC:CH:DCP[a]	800	6.0
REV	HPC:CH:DCP[a]		3.7
	OPC:CH:DCP[a]		3.0
	EPC:CH:DCP[a]		7.4

Composition of liposomes: APC:CH, 1:1; APC:CH:DCP 1:1:0.2 or [a]1:1:0.25.

Electron microscopy

The size and structure of the vesicles were estimated by negative staining using uranyl acetate as described by Arndt.[28]

Fluorimetry

Fluorescence measurements were performed using a spectrofluorimeter MK2 (Farrand) at an excitation wavelength of 490 nm and an emission wavelength of 520 nm. The total amount of CF was determined by destroying the liposomes with Triton X-100 (final concentration: 1% detergent). The encapsulation capacity (*e*) and CF leakage (*f*) were estimated according to Nuhn.[29] No correction was made to take into account the change in intra-vesicle self-quenching when large amounts of CF had leaked out.

In vitro experiments

Direct cytotoxicity assay. Human mouth epidermoid carcinoma cells (KB; ATCC, CCL 17) were used in passage 16–18, and human skin and muscle fibroblasts (M22; GUS, Moscow) were used in passage 2–5. The cells were dispersed from confluent cultures by 0.5% trypsin and 0.05% versene. Eagle's modified essential medium 1959 plus 10% FCS (Staatliche Immunpräparate und Nährmedien, Berlin, Germany) and standard antibiotics were used. Test plates (Linbro, Flow Laboratories, Irvin, Scotland) for drug testing were prepared with 1×10^5 (KB) or 2×10^5 (M22) cells/well. Cells counts were performed in a Bürker hemocytometer chamber. Cells (standard curve, 200 μl cell suspension, 2.5×10^3 to 10×10^5 cells/well) were cultured for 24 h at 37°C in a humidified 1.54% CO_2 atmosphere. Cells were incubated with different concentrations of free or liposomal alkylphosphocholine (indicated in Table 4 and Figure 3) for 30 and 72 h, respectively. Cell counts were performed in a hemocytometer chamber. A 200 μl aliquot of cell suspension ($2.5–10 \times 10^4$ cells/well) was added for the standard curve. Viable cells were counted in a modified crystal violet assay[30] by spectrophotometric measurement at 590 nm.

Tumor cell growth inhibition assay. Tumor cell growth inhibition was measured by the decrease of [^{3}H]thymidine incorporation into the target cells. MethA sarcoma cells in ascitic form were harvested from BALB/c mice, suspended in PBS and concentrated by centrifugation. Then, 96-well microtiter plates (Falcon, Becton Dickinson and Company, Lincoln Park, USA) were prepared with 5×10^4/well MethA sarcoma cells in RPMI medium plus 10% FCS and standard antibiotics. Tumor cells were incubated with different concentrations of alkylphosphocholines or liposomes in PBS for 18 h. The tumor cells were then pulsed with [^{3}H]thymidine (20 μl, 54 μCi/ml). After another 18 h of incubation, the cells were collected on a paper filter. After washing and drying, the radioactivity was measured by liquid scintillation counting. The results are given as percent inhibition of [^{3}H]thymidine incorporation related to untreated tumor cells. All determinations were done 8-fold in at least three independent experiments.

Statistics

Student's *t*-test[31] was used for statistical evaluation.

Results

Characterization of alkylphosphocholine liposomes

Table 2 shows type, lipid composition, size and encapsulation capacity of liposomes made of HPC, octadecylphosphocholine (OPC) or eicosanylphosphocholine (EPC). Especially in the presence of dicetylphosphate the whole amount of administered lipids is converted in liposomes. Mixtures of alkylphospholipids and cholesterol without a charged constituent form liposomes and aggregates of unknown structure (data not shown).

The efficiency of entrapment of CF in liposomes (SUV, MLV, REV) formed from acetylphosphocholine, CH and dicetylphosphate is about 0.5–1% for SUV and 1–4% for MLV. As expected, REV have the highest encapsulation capacity. The entrapment for the fluorescent dye is of the same magnitude as that known for natural phospholipids.[23]

Figure 1 shows the percentage CF released from SUVs of different lipids incubated in a PBS buffer

Table 3. Drug entrapment as a function of lipid composition and preparation technique

Lipid composition	Drug	Encapsulation capacity (% of total)
HPC:CH:DCP	daunorubicine	48
OPC:CH:DCP	daunorubicine	45
HPC:CH:DCP	mitoxantrone	>95
HPC:CH:DCP	mitoxantrone	75–88[a]
EggPC:CH:DCP	daunorubicine	70
Hydrogenated EggPC:CH:DCP	mitoxantrone	>98

[a]pH gradient technique; starting alkylphosphocholine concentration 29 mmol. Molar ratio: alkylphosphocholine:drug, 10:1; lipid:CH:DCP, 1:1:0.25.

(pH 7.4, 4°C). Liposomes made from different acetylphosphocholines were more stable than vesicles from egg phosphatidylcholine. Surprisingly, SUV show a very slow leakage rate of the entrapped marker (data for MLV not shown). Table 3 demonstrates that mitoxantrone can be accumulated into SUV which exhibit a transmembrane pH gradient.[20] The encapsulation efficiency is nearly 100%.

In vitro test

All alkylphosphocholines show a clear cytotoxic activity against the investigated KB and M22 cells, depending on the number of carbon atoms in the alkyl chain, concentration and incubation time. The results (Table 2 and Figure 1) demonstrate that the activity of the C_{16} compound was lower than that of the C_{18} and C_{20} compounds, whereas the difference of cytotoxic activity between the last two compounds was not so significant. The concentration needed for complete inhibition of KB tumor cell growth was approximately 25 μg/ml. Amounts of 5 μg led to an inhibition of approximately 80% (compared with control) for OPC and EPC, and only to 50% for HPC. The same dependency on alkyl chain length was found with M22 cells as target cells; however, the concentrations of alkylphosphocholines that were needed to induce a comparable effect were 5- to 10-fold higher. The IC_{50} and the IC_{90} values are given in Table 4.

Tumor target cells were incubated for 30 and 72 h with different concentrations of alkylphosphocholines in free or liposomal form (Table 5). Alkylphosphocholines both in a free and liposomal form decreased the number of KB cells at both

Table 4. Cytotoxic effects of alkylphosphocholines on human mouth epidermoid carcinoma cells (KB) and human skin and muscle fibroblasts (M22)

	IC_{50}		IC_{90}	
	KB	M22	KB	M22
HPC	3.75	39.0	>25	>100
OPC	2.05	13.0	>25	>50
EPC	1.35	8.0	>25	>25

Either 10^5 (KB) or 2×10^5 (M22) cells/well were incubated with different concentrations of alkylphosphocholine for 30 h. All values are given in μg/ml for the concentration necessary to kill 50 or 90% of the cells.

Table 5. Cytotoxic effects of alkylphosphocholines or liposomes of alkylphosphocholines[a] on human mouth epidermoid carcinoma cells (KB) and human skin and muscle fibroblasts (M22)

Lipid and final concentration[b] (mmol)	30 h				72 h			
	KB		M22		KB		M22	
	free	lip	free	lip	free	lip	free	lip
HPC[b]								
1×10^{-2}	—	—	79.1[c]	31.3[d]	—	—	69.0	13.4[d]
2×10^{-3}	—	—	95.4	82.3	—	—	99.1	67.9[d]
6.8×10^{-5}	44.7[c]	8.3[d]	—	—	42.4	5.03[d]	—	—
6.8×10^{-6}	91.1	54.33[d]	—	—	85.0	11.73[d]	—	—
OPC[b]								
1×10^{-2}	—	—	31.9	21.7	—	—	13.4	4.3
2×10^{-3}	—	—	91.8	83.8	—	—	82.0	57.0[d]
6.8×10^{-5}	25.0	6.13[d]	—	—	27.8	6.93[d]	—	—
6.8×10^{-6}	82.0	21.93[d]	—	—	85.0	11.73[d]	—	—
EPC[b]								
1×10^{-2}	—	—	20.8	22.4	—	—	6.7	8.3[d]
2×10^{-3}	—	—	99.7	94.4	—	—	72.4	46.7[d]
6.8×10^{-5}	38.1	4.5[d]	—	—	23.6	3.9	—	—
6.8×10^{-6}	92.7	28.9	—	—	89.4	16.1[d]	—	—

[a]Composition: APL:CH:DCP, 1:1:0.2, molar ratio.
[b]1×10^{-2} mmol:HPC, 4.07 μg/ml; OPC 4.36 μg/ml; EPC, 4.65 μg/ml.
[c]The cell growth as a percentage of the control is given, all determinations were done in triplicate.
[d]Significantly different to free etherlipid, $p < 0.005$.

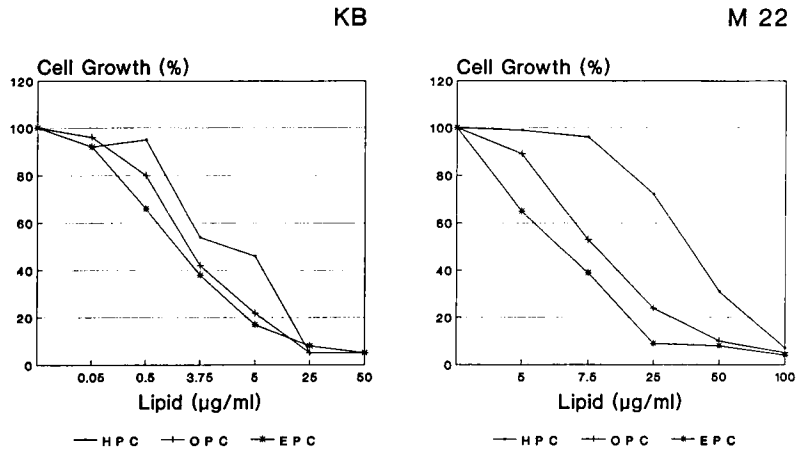


Figure 2. Direct cytotoxicity of alkylphosphocholines. Human mouth epidermoid carcinoma cells (KB) and human skin and muscle fibroblasts (M22) were incubated with different concentrations of alkylphosphocholines for 30 h as indicated in Materials and methods. The mean percentages of cell growth compared to the control of at least three independent experiments in triplicate are given.

incubation times. Concentrations as low as 5 µg/ml of HPC, 2.5 µg/ml of OPC and 0.5 µg/ml of EPC in the liposomal form were needed to give more than 90% inhibition of KB cells after 72 h of incubation time.

A significant difference in cytotoxic activity against KB tumor cells was found by comparing the free ether lipids and liposomes prepared from equimolar amounts of these alkylphosphocholines. All liposomal preparations showed a stronger cytotoxic effect. Generally, the liposomes composed of EPC showed the strongest effects at all concentrations investigated. Furthermore, a longer incubation time increased the differences in inhibition between liposomal and free alkylphosphocholine.

The same results were found for the M22 cells, but the concentrations needed for 50% inhibition were much higher (Table 5).

The cell growth inhibition of MethA sarcoma cells by alkylphosphocholines showed that this effect is strongly related to increasing chain length. With EPC, only 25 µg/ml was needed to induce 90% growth inhibition; the same effect was obtained with 50 µg/ml of OPC. HPC was the least active compound in this assay (Figure 2).

In contrast, in the cytostatic assay with MethA cells the liposomal alkylphosphocholines are significantly less toxic as compared with the free substances (Figure 3).

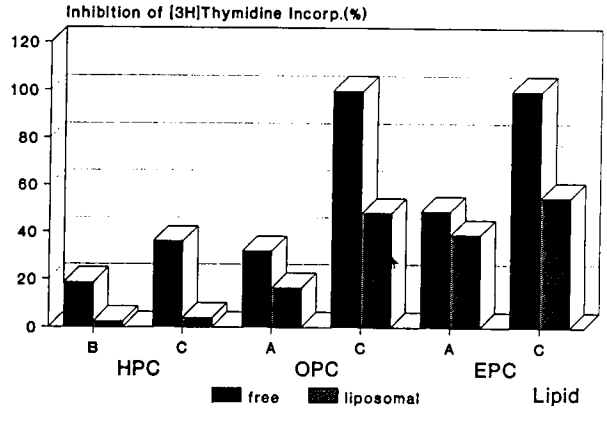


Figure 3. Cytostatic activity of alkylphosphocholines. MethA sarcoma cells (5×10^4 per well) were incubated with different concentrations of alkylphosphocholines and MLV made of alkylphosphocholines for 24 h as indicated in Materials and methods. The used concentrations of alkylphosphocholines are: (A) 13 nmol/ml, (B) 65 nmol/ml and (C) 130 nmol/ml. The mean percentages of cell growth inhibition compared to the control of at least three independent experiments, performed 8-fold, is given.

Discussion

Lipids like phosphatidylcholine which form liposomes normally consist of two hydrophobic fatty acid chains in the glycero backbone and a hydrophilic head group. Lysolecithin or substances with a lysolecithin-like structure normally disturb the bilayer structure of such liposomes.[32]

Our experiments demonstrate that alkylphospholipids form liposomes in the presence of CH and a charged amphiphil, like dicetylphosphate or

dipalmitoyl-phosphatidylglycerol. In particular, the SUV show remarkable stability. We assume that a more tightly packed (rigid) bilayer, stronger intrabilayer attractive dispersion forces and a better packing in the polar head group domain are the reasons for that increased stability. Detailed examinations are in progress.

Similar to vesicles composed of conventional phospholipids, e.g. phosphatidylcholine, liposomes built up from alkylphospholipids, CH and a charged amphiphil are suitable carriers for different compounds. We encapsulated CF and the anticancer drugs mitoxantrone[21] and daunorubicine in such vesicles with entrapment efficiencies comparable with common liposomes.[29]

A lot of investigations on physiological, especially the cytotoxic, tumoricidal and therapeutic, effects of alkylphosphocholines have been described, most of them focused on HPC.[12–15] Only a small number also included the C_{18} compound (OPC).[7,12,21] For the evaluation of the influence of the chain length in biological effects, we used the HPC, OPC and EPC (C_{20}) in our studies.[21]

We found that with an increase in chain length, the antiproliferating, cytotoxic and activating properties increased. This is in agreement with results from other groups demonstrating the same tendency by comparing HPC with OPC *in vitro* and *in vivo*.[12,21] To what extent this is related to the change of the amphipathic properties still remains unclear. Other data leave doubt about such a simple explanation. There are differences between the cytostatic activity in different cell lines when using the same lipids, or a loss of activity is caused by changing the structure only minimally.[12,33]

The etherlipids used are cytotoxic within a concentration range between 5 and 100 nmol/ml, which is comparable with concentrations used to inhibit HL60, U 937 or Raji leukemic cells.[12]

Incubation of MethA or C26 tumor cells with liposomes composed of alkylphosphocholines, CH with or without negatively charged lipid decreased the cytotoxic effects of the incorporated etherlipids compared with equimolar free compound. Alkylphosphocholine liposomes have only a slight inhibitory effect on MethA sarcoma cells and no cytotoxic effect on C26 colon carcinoma cells (data not shown). The minor effects that we observed in our experiments with MethA cells may be related to free etherlipid which had not been incorporated into the liposome layer. Furthermore, turbidity measurements showed that the stability of liposomes without a charged carrier (MLV, APC:CH 1:1) decreased with time (data not shown).

The reduced or lacking cytotoxic effects of vesicles demonstrate that the effect of etherlipids on tumor cells is related to the drug structure and requires the free or micellar form of alkylphosphocholine. The interaction between the etherlipid and the tumor cell wall is complicated, in the case when the lipid is incorporated within the liposomal layer.

In what way a direct interaction with the cell wall or a platelet aggregation factor related receptor mediated effect on the tumor cells is the reason for cytotoxic results is under discussion.[33,34]

Apart from a 10-fold higher sensitivity to alkylphosphocholine, KB cells and M22 fibroblasts were also more sensitive to alkylphosphocholine liposomes as compared with equimolar mixtures of free etherlipid than MethA cells and C26 carcinoma cells. The influence of the chain length was the same as found in our other experiments.

One possibility to explain this is to assume a different mechanism of interaction between etherlipid liposomes and the membranes of KB cells and M22 fibroblast cells compared with those of MethA cells. It may be possible that the high toxicity of alkylphosphocholine liposomes against the KB and M22 cells is due to an ability of these cells to internalize the vesicles by endocytosis. This mechanism was found to partly play a role in etherlipid–tumor cell interaction.[35,36] In particular, large particles are favored for internalization by endocytosis, due to this fact liposomes should be better endocytosed than the small free etherlipid molecules.

Another explanation is based on the findings that the liposome constituent CH modulates alkylphospholipid uptake and cytotoxicity.[37,38]

Investigations on the influence of liposomes on tumor cell response are being continued.

Acknowledgment

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