J. Membrane Biol. 182, 61–69 (2001)
DOI: 10.1007/s00232-001-0027-z

The Journal of
Membrane
Biology

The Composition-Dependent Presence of Free (Micellar) Alkylphospholipid in Liposomal Formulations of Octadecyl-1,1-dimethyl-piperidino-4-yl-phosphate Affects its Cytotoxic Activity in vitro

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Received: 4 October 2000/Revised: 29 March 2001

Abstract. This study was performed to investigate the effect of cholesterol content, surface charge and sterical stabilization on the physico-chemical properties of liposomes prepared from the cancerostatic alkylphospholipid, octadecyl-1,1-dimethyl-piperidino-4-yl-phosphate (D21266), and their relationship to in vitro cytotoxicity. Stable incorporation of OPP into liposomes was found to be highly dependent on the cholesterol content. ^{31}P-NMR spectroscopy as well as analysis of the lipid composition of OPP-containing liposome formulations revealed an increase in the amount of non-liposome-associated, micellar OPP as the cholesterol content decreased. The fraction of non-liposome-associated OPP constituted about 10% of total OPP when cholesterol was present in equimolar amounts (45.5/45.5 mol %) and increased to approximately 30% at a twofold excess of OPP over cholesterol (58.8/29.4 mol %). In monolayer incorporation studies it was shown that the existence of an increasing micellar pool of lipids leads to increased lipid transfer into the target monolayer. Liposome formulations containing more OPP than cholesterol were also found to display greater cytotoxicity. However, all liposome formulations were less cytotxic than pure (micellar) OPP. Cytotoxicity was not affected by the incorporation of *N*-methoxy-polyethyleneglycol$_{2000}$-phosphoethanolamine, a lipid that is known to reduce liposome uptake into phagocytic cells.

The results demonstrate that the increase in cell toxicity correlates with the increase in non-liposome-associated, micellar OPP, which can readily exchange into cellular membranes.

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Key words: Liposomes — Alkylphospholipid — Monolayer — Cytotoxicity – Breast cancer — Micelles

Introduction

Liposomes are widely used as carriers for antibiotic, antifungal and anticancer drugs as well as for genetic material such as plasmid DNA and antisense oligonucleotides. Apart from being a carrier, vesicles can also be directly prepared from mixtures of cancerostatic lipids including etherlipids and alkylphospholipids with other lipids (Zeisig et al., 1993, 1998; Kaufmann et al., 1996; Perkins et al., 1997; Zeisig, Fichtner & Arndt, 2000). Alkylphospholipids (APLs) such as octadecyl-1,1-dimethyl-piperidino-4-yl-phosphate (D21266; OPP) have a single alkyl chain, which is directly linked to a phosphate-containing head group, whereas etherlipids, including alkylphosphatidylcholines, have a glycerol backbone to which the alkyl chain is attached via an ether bond. The lack of the glycerol backbone results in a decrease of the headgroup area relative to the hydrocarbon cross-sectional area. As a consequence, OPP forms a narrower inverted cone than the corresponding alkylphosphatidylcholines. These structural differences could affect the physicochemical and biological properties of the free compounds as well as the characteristics of APL-containing liposomes.

APLs were shown to exhibit a strong antitumor effect, in particular against human breast cancer cells (Fichtner et al., 1994; Terwogt et al., 1999). Attempts to use APL-liposomes or even free alkylphospholipids as immunomodulating agents with the aim to obtain an enhanced antitumor effect based on effects observed with etherlipids (Berdel et al., 1981), failed even though some

macrophage activating effects were observed in vitro (Zeisig et al., 1995). In addition, APLs are detergents and also show strong membrane-lytic/hemolytic activity. The successful use of APLs as pharmaceutical agents requires a reduction of these unwanted nonspecific toxic effects while still maintaining appropriate levels of specific biological activity. Lysolipids and monoalkyletherlipids can form liposomes in combination with lipids of complementary shape such as cholesterol and DOPE. Bilayer incorporation was shown to strongly reduce their hemolytic activity. Up to now, sterically stabilized liposomes containing the APL derivative OPP have been found to be the most toxic APL-liposome formulation against breast cancer cells in vitro and in vivo (Zeisig et al, 1998), whereas the effect on macrophages was negligible (Zeisig et al., 1995).

Despite these results, an optimization of the liposome formulations was necessary, which requires knowledge about the interrelationship between the physicochemical characteristics and the cytotoxic properties. The aim of this study was, therefore, to investigate the effect of lipid composition on the physicochemical properties of OPP-containing liposome formulations in order to obtain a better understanding of the factors that determine biological activity.

Materials and Methods

Materials

Octadecyl-1,1-dimethyl-piperidino-4-yl-phosphate was a generous gift from Dr. Hilgard (ASTA Medica, Frankfurt, Germany). Dicetylphosphate (DCP) and calcein were purchased from Serva (Heidelberg, Germany). 1-Palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC), 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoethanolamine (POPE) and 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoserine (POPS) were obtained from Avanti Polar Lipids (Alabaster, AL) and cholesterol (CH) from Calbiochem (La Jolla, CA). 1,2-distearoyl-sn-glycero-3-[*N*-methoxy-(polyethylene-glycol$_{2000}$)]-phospho-ethanolamine (PEG-DSPE) was purchased from Sygena (Liestal, Switzerland). The tetrazolium dye, MTT, was obtained from Sigma-Aldrich (Steinheim, Germany), Sephadex G50 from Pharmacia (Uppsala, Sweden) and HPTLC plates from Merck (Darmstadt, Germany). Triton X-100 was a product of Ferak (Berlin, Germany). Dichloromethane and methanol from Merck (Darmstadt, Germany) were used in Lichrosolv® gradient grade quality. Phosphate buffered saline (PBS, pH = 7.5), L-glutamine and fetal calf serum (FCS) were products of Gibco, Life Technologies (Eggersstein, Germany) and penicillin and streptomycin of Biochrom KG (Berlin, Germany).

Liposome Preparation

Liposomes were prepared from multilamellar vesicle (MLV) suspensions as described previously (Zeisig et al., 1998). Briefly, lipid films consisting of the lipid mixtures listed in Table 1 were hydrated in PBS buffer to obtain the MLV. Large unilamellar liposomes were generated from these MLVs by repeated extrusion through 100 nm polycarbonate filters using a LiposoFast Basic System (Avestin, Ottawa, Canada). The initial OPP/CH ratio was varied from 1/0.5 to 1/1 (mol/mol) with CH comprising 27.8% to 45.5% of the total lipid. Negatively charged liposomes were prepared with dicetylphosphate (DCP) and positively charged liposomes with dimethyl-dioctadecylammonium bromide (DDAB). Liposome size was measured by dynamic light scattering using a Coulter Counter N4 MD (Coulter Electronics, Hialeah, USA). Liposomes were used if size was about 150 nm in diameter with a polydispersity index lower than 0.25.

Determination of Lipid Concentrations

OPP and CH concentrations were determined by HPTLC using $CHCl_3$/MeOH/acetic acid/water (50/30/8/4, v/v) or $CHCl_3$/Hexan/MeOH (49/49/2), respectively, as mobile phase. The plates were dried at 140°C for 10 min and developed by dipping into a $MnCl_2$ solution (0.4 g $MnCl_2$ in 120 ml 50% methanol and 4 ml H_2SO_4) for 5 sec followed by 7 min of heating at 140°C to visualize the lipid spots. Quantification was performed relative to standard curves covering lipid concentrations from 0.5–1.5 nmol/spot by scanning of the plates at 564 nm using a Camag scanner II (Camag, Muttenz, Switzerland). The lipid concentrations given in this study represent the actual OPP concentration in the formulation. In some instances, phospholipid concentrations (total lipid) were quantified by a phosphorus assay (Marinetti, 1962).

Zeta-potential Measurements

Electrophoretic light scattering measurements were performed with a Coulter Delsa 440SX (Coulter Electronics). The zeta-potential of LUVET, diluted with PBS to a concentration of about 0.5 mg total lipid/ml, was determined at 5 different angles at 25°C. All measurements were done in triplicate on two different liposome preparations. The mean zeta potentials (mV) are listed in Table 1.

Separation of Liposomes from Non-Liposome-Associated OPP

OPP-MLV, 1 ml (10 mM) of different compositions (for details *see* Table 1 and 2) were subjected three times to centrifugation at 100,000 × g for 30 minutes using a Beckman L70 ultracentrifuge. After each run the supernatants containing the micellar OPP and very small vesicles were removed and the pellets resuspended in PBS. The final suspensions were employed after appropriate dilution for quantification of OPP and CH by HPTLC as described above. LUVET dispersions were applied in aliquots of 500 µl (10 mM OPP) to 20 × 1 cm Sephadex G50 columns equilibrated in PBS. 1.0 ml fractions were collected and the peak fraction was analyzed for cholesterol and OPP content as described above.

^{31}P-NMR Spectroscopy

^{31}P-NMR spectra were measured with a Bruker MSL200 spectrometer operating at 81 MHz. Free induction decays corresponding to 3200 or 6400 scans were obtained by using a 3 µsec 55° pulse with a 1 sec interpulse delay and a spectral width of 10,000 Hz. All measurements were performed at 25°C on a 1.5 ml sample in a 10 mm probe. Protons were decoupled. An exponential multiplication corresponding to 5 Hz of line broadening was applied to the FIDs prior to Fourier transformation. All samples contained 10 mg of total lipid.

Solute Retention Capacity in Serum-Containing Buffer

Serum-induced leakage of the fluorescent dye calcein, entrapped at self-quenching concentrations in OPP liposomes, was followed by monitoring the dequenching of the calcein fluorescence. LUVETs were prepared by hydration of a lipid film with an aqueous solution containing 100 mM calcein adjusted to pH 7.5 by addition of sodium hydroxide, followed by extrusion through 2 stacked 100 nm filters. Unentrapped calcein was exchanged against PBS by size exclusion chromatography on a Sephadex G50 column. The resulting liposome solution was diluted to a OPP concentration of 100 μM in 50% fetal calf serum/PBS and incubated at 25°C for 48 hours. Then 100 μl aliquots were applied to a 96-well plate (Maxisorp black, Nunc, Kamstrup, Denmark). The fluorescence at 510 nm was measured with a SLT Fluostar plate reader (TECAN, Crailsheim, Germany) with the excitation wavelength set to 485 nm. Calcein leakage was calculated according to % leakage $= (F_s - F_b)/(F_{Tx} - F_b) \cdot 100$, where F_s is the fluorescence of the sample, F_b the background corresponding to calcein containing liposomes and F_{Tx} the Triton X-100 value. The value for 100% leakage (maximum dequenching) was obtained by addition of 50 μl of a 10% Triton X-100 solution. The trapped marker was expressed as the molar amount of calcein/OPP.

Monolayer Experiments

The transfer of lipids into a monolayer can be followed in Langmuir experiments accordingly to Seelig and Macdonald (1989). A rectangular Teflon trough (type Biotrough, Riegler and Kirstein, Wiesbaden, Germany) with a total area of 62 cm^2 was used. The surface pressure was measured with a Wilhelmy balance connected to a Whatman No. 1 filter paper. A mixture of POPC:POPE:POPS:CH (4:2:1:3.6 (mol/mol), 7.1 nmol total lipid) was dissolved in chloroform and spread on the buffer surface (PBS pH 7.5, 25°C) to form a monolayer, which was then compressed to 25 mN/m. This surface pressure was maintained throughout the experiment by means of an electronic feedback system. The liposome formulations (25 nmol OPP) were injected into the stirred subphase and left to equilibrate between the subphase and the monolayer. The area increase as a result of the insertion of lipid molecules from the OPP liposomes into the target lipid monolayer was expressed as $\Delta A/A_0$ [%] (Seelig and Macdonald, 1989). All experiments were done in triplicate.

Determination of in vitro Cytotoxicity

The estrogen receptor-negative human breast cancer cell lines MT1 (Naundorf, Fichtner et al., 1994) and SKBR-3 [ATCC, Parklawn, USA] were cultured in antibiotics-containing RPMI-1640 media supplemented with 2 mM L-glutamine and 10% heat-inactivated fetal calf serum. Cells harvested in the exponential growth phase were seeded at a density of 2×10^4/well in a 96-well microtiter plate (Falcon) one day before the experiment. OPP-containing liposome dispersions as well as OPP solutions were serially diluted with media to concentrations covering a range from 12.5–200 μM and applied to the cells. After 24 hr cells were washed twice with PBS and incubated with 0.5 mg/ml MTT in RPMI for 4 hr. Finally, the supernatant was removed, the formed formazan dissolved in isopropanol/HCl and quantified by absorption measurements at 530 nm using an SLT-plate reader (TECAN Germany, Crailsheim, Germany). Growth inhibition was expressed relative to untreated control cells. The IC_{50} data were determined from the growth inhibition curves obtained from 6 different dilutions each done in triplicate. IC_{50} data represent the average of at least 4 independent experiments.

Statistics

Statistical comparisons of in vitro data were performed with the unpaired Student's *t*-test (two populations). Differences were considered to be significant at $P < 0.05$.

Abbreviations

The abbreviations used are: APL, alkylphospholipid; CH, cholesterol; DCP, dicetylphosphate; DDAB, dimethyldioctadecylammonium bromide; IC_{50}, concentration of drug required to inhibit cell growth by 50%; LUVET, large unilamellar vesicle made by extrusion technique; MLV, multilamellar vesicle; NBD-PE, 1,2-dioleoyl-sn-glycero-3-[*N*-(7-nitro-2-1,3-benzoxadiazol-4-yl)]-phosphoethanolamine; OPP, octadecyl-1,1-dimethyl-piperidino-4-yl-phosphate; PBS, phosphate buffered saline; PEG-DSPE, 1,2-distearoyl-sn-glycero-3-[*N*-[methoxy-(polyethyleneglycol)-2000]-phosphoethanolamine; POPC, 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine; POPE, 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoethanol-amine; POPS, 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoserine; TM, target membrane.

Results

Physicochemical Properties of OPP-Containing Liposome Formulations

Table 1 lists lipid compositions and physicochemical characteristics of the OPP-containing liposome formulations used in this study. A parameter, which is important for the interaction of liposomes with cells, is the surface charge of the liposomes which strongly affects the uptake by cells. Zeta potentials are a convenient measure of the surface charge and are listed in Table 1 for all liposome formulations. The liposome formulations have zeta potentials of similar magnitude with the absolute sign of the surface charge being the main difference. The presence of PEG-DSPE leads to a clear decrease in zeta potential.

The OPP-containing liposome formulations were further characterized with respect to their ability to entrap and retain entrapped solutes. The amount of the aqueous trap marker calcein, associated with the OPP-containing liposome formulations, decreased by a factor of 3–4 when the OPP content was increased from 45.5% to 58.8% and did not depend on the sign of the surface charge (Table 1). This could be a consequence of a decrease in the number of liposomes, of a reduction in liposome size or due to increased membrane permeability. The latter possibility was ruled out by the observed decrease in serum-induced leakage of entrapped calcein with increasing OPP content (Table 1). Thus, liposome formulations containing equal amounts of OPP and cholesterol exhibit much higher trapped volumes than lipo-

Table 1. Composition of OPP-liposomes and their stability in serum

Type	Name	Composition				ζ-Potential	Stability in serum[a]	
		Molar ratio[b] OPP/CH/X/PEG_{2000}DSPE	OPP	CH [% of total lipid]	Charged compound	[mV]	Marker content [mmol/mol OPP]	Marker release [% of total marker]
Negative[c]	Neg10	10: 10:2:0	45.5	45.5	9.09	−58.8 ± 6.8	87.5 ± 2.6	63.4 ± 7.7
	Neg7.5	10:7.5:2:0	51.3	38.5	10.26	−66.2 ± 9.9	71.5 ± 6.1	53.5 ± 10.0
	Neg5	10: 5:2:0	58.8	29.4	11.76	−65.5 ± 8.4	30.4 ± 4.1	44.3 ± 6.3
	Neg5P	10: 5:2:1	55.6	27.8	11.11	−27.3 ± 6.4	44.0 ± 1.7	32.9 ± 4.4
Positive[d]	Pos10	10: 10:2:0	45.5	45.5	9.09	49.2 ± 5.6	90.2 ± 5.1	83.1 ± 13.5
	Pos7.5	10:7.5:2:0	51.3	38.5	10.26	66.2 ± 10.6	91.7 ± 4.0	48.6 ± 5.5
	Pos5	10: 5:2:0	58.8	29.4	11.76	52.1 ± 5.4	22.4 ± 3.6	47.5 ± 2.8
	Pos5P	10: 5:2:1	55.6	27.8	11.11	30.9 ± 6.8	39.2 ± 5.3	39.5 ± 4.0
Target	TM	40:20:10:36:0.5:0.5[e]		33.6	65.4[f]			n.d.

Given are the molar parts of the components for each liposomal preparation used in this study. Lipsomes were usually prepared in a concentration of 10 μmol OPP/ml as described in Materials and Methods and diluted before use to appropriate concentrations.
Abbreviations used for liposomes in Tables 1 and 2 and in Figs. 1 and 3:
Neg10 (7.5, 5): Negatively charged liposomes with 10 (7.5, 5) molar parts of CH;
P: Sterically stabilized liposomes
Pos: Positively charged liposomes
TM: Target membrane
[a] Characterized by release of encapsulated marker calcein after storage in FCS/PBS (1/1). Calcein was determined by fluorescence measurements after 48 hours as described in Materials and Methods.
[b] Liposomal composition OPP/CH/X/PEG_{2000}DSPE where x is a charged component.
[c] Charged component x is dicetylphosphate (DCP)
[d] Charged component x is dioctadecyl-dimethylamine bromide (DDAB)
[e] POPC/POPE/POPS/CH/Rho-PE/NBD-PE
[f] Ratio of (POPC/POPE/POPS together)/CH
n.d. not determined

some formulations containing an excess of OPP over cholesterol, but appear to be less stable in serum than the latter. For example, for negatively charged vesicles, a reduction of marker release was observed from 63 down to 32% with an increase of OPP in the liposomal membrane (Table 1). In addition, the absolute amount of released marker was linearly dependent on the cholesterol amount in the initial formulation. Sterical stabilization or charge had no effect on marker release.

Effect of the Cholesterol Content on Stable Incorporation of OPP into Liposomes

The ability of cholesterol to stabilize OPP into a bilayer organization was investigated in the following. In a first set of experiments MLVs were prepared containing OPP and cholesterol, in molar ratios between 1–2, and a small amount of the negatively charged lipid DCP (*see* Table 1). The structure of the lipid dispersions was characterized by ^{31}P-NMR. The asymmetric line shape seen in Fig. 1, with a low-field shoulder and a high-field peak and a chemical shift anisotropy of approximately 30 ppm, corresponds to phospholipid organized in extended bilayers (Brown & Seelig, 1978; Cullis & de Kruijff, 1979). Superimposed on this spectrum are two peaks of much smaller line width centered around 0 ppm (*arrows*

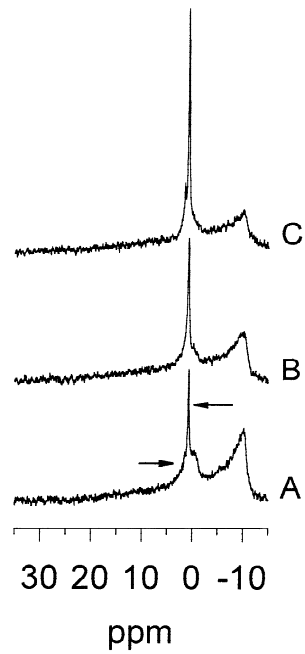

Fig. 1. ^{31}P-NMR spectra of MLVs containing different molar ratios of OPP to cholesterol. (*A*) OPP/CH/DCP (10:10:2 molar ratio; Neg10), (*B*) OPP/CH/DCP (10:7.5:2; N7.5) and (*C*) OPP/CH/DCP (10:5:2; Neg5). Each sample contained 10 mg of lipid. A line-broadening of 5 Hz was applied prior to Fourier transformation.

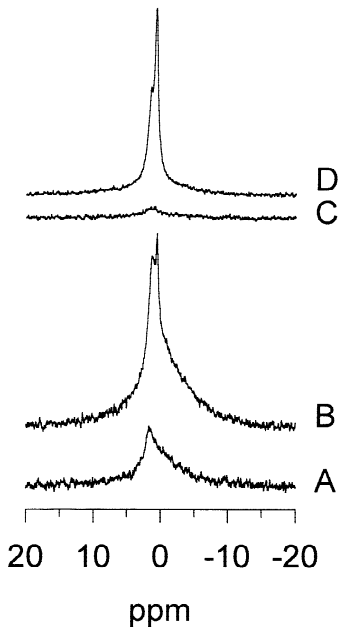

Fig. 2. ^{31}P-NMR spectra of extruded liposomes containing different ratios of OPP to cholesterol. OPP/CH/DCP (10:10:2 molar ratio) liposomes in the presence (*A*) and absence of 5 mM Mn^{2+} (*B*); and OPP/CH/DCP (10:5:2; Neg5) liposomes in the presence (*C*) and absence of 5 mM Mn^{2+} (*D*). The lipid concentration was 10 mg.

in Fig. 1). It is known that the chemical shift anisotropy (line width and asymmetry) decreases with decreasing size (increasing tumbling rate) of the lamellar systems (Burnell, Cullis & de Kruiff, 1980). The broader peak can, therefore, be assigned to larger liposomes. Narrow isotropic ^{31}P-NMR spectra are observed for small vesicles (SUV) and micelles where rapid tumbling of the vesicles or micelles averages the chemical shift anisotropy (Cullis & de Kruijff, 1979; Yeagle, 1996). The line width of 0.3–0.36 ppm (25–30 Hz) of the narrow isotropic signal is consistent with the presence of small micelles. In micelles all phosphate groups are exposed to the bulk aqueous solution and are, therefore, accessible to line broadening agents such as Mn^{2+}. Addition of 5 mM Mn^{2+} to the MLV dispersions resulted in the disappearance of the narrow isotropic peak. This further confirms its micellar origin. The size of the narrow isotropic signal increased with increasing OPP content, indicating that more OPP is in micellar form. Upper estimates of 11%, 19% and 25% were obtained from planimetry of the respective spectra. Fig. 2 shows the ^{31}P-NMR spectra of the liposomal dispersions with the highest and lowest OPP content used in the previous experiments after extrusion through filters of 100 nm pore size. The line width as well as the appearance of the spectra changes with the OPP-to-cholesterol ratio. Both spectra have two peaks, one of which is slightly downfield-shifted (to the left-hand side) with respect to the other. Chemical shift differences can arise from different phospholipid species (OPP and DCP) or from phosphate groups in different environments (micelles, liposomes, etc.). The right-hand peak has the same chemical shift as the narrow isotropic peak seen in the MLV spectra (Fig. 1). Based on this and on the increase in its contribution with increasing OPP content, it may be speculated that it represents OPP micelles. It should also be noted that the width of the spectrum is much smaller for the formulation with the higher OPP content. This indicates a decrease in size and/or number of the liposomes and agrees with the observed decrease of the trapped volume (Table 1). Upon addition of Mn^{2+}, about 65% of the signal is lost in the sample containing 45.5% OPP (OPP/CH = 1), while 85% of the lipid phosphate groups are accessible to Mn^{2+} when OPP is present at 58.8% (OPP/CH = 2).

In a second set of experiments extruded liposome dispersions were fractionated by centrifugation (MLV) or by size exclusion chromatography (LUVET) to determine the extent of bilayer incorporation of OPP. Table 2 shows that already the formation of MLV was incomplete with a deviation of about 10% from the initial OPP/CH ratio. For LUVET this ratio decreased by 12% and 31%, respectively, as the OPP content increased from 45.5% to 58.8%. The decrease in OPP/CH ratio was smaller for the liposome formulations containing PEG-DSPE and the positively charged lipid DDAB.

A final set of experiments demonstrates the ability of OPP to exchange from OPP-containing liposome dispersions into a target membrane. The presence of exchangeable OPP, that is OPP in micellar, not liposome-associated form, as well as OPP that is not stably incorporated in liposomes, can be detected by surface pressure measurements (Punnonen et al., 1989). A lipid monolayer consisting of POPC:POPE:POPS:CH (4:2:1:3.6 mol/mol) was spread on the buffer surface in a Langmuir trough. The liposome formulations (25 nmol OPP) were injected into the subphase and left to equilibrate. Transfer of lipid from the subphase into the target monolayer results in an increase in monolayer surface area. Fig. 3 shows that lipid transfer progressively increased as the cholesterol content decreased. The increase in surface area relative to OPP micelles, which caused an area increase of 87.2%, was about 70% lower for liposome formulations containing 45.5% OPP (*Neg10*) and about 45% lower for liposome formulations containing 58.8% OPP (*Neg5*). There was no difference between negatively (*Neg10*) and positively charged (*Pos10*) liposomes. The largest increase in surface area was obtained for the PEG-DSPE containing Neg5p formulation.

Effect of Lipid Composition on Cell Cytotoxicity

The liposome formulations were investigated with respect to their ability to inhibit the growth of two different breast cancer cell lines, MT1 and SKBR-3. Micellar

Table 2. Changes in molar ratio of OPP/CH after centrifugation (MLV) or size exclusion chromatography (LUVET)

Formulation	Molar ratio of OPP/CH				
	Before treatment[a]	After centrifugation		After extrusion	
	Ratio	Ratio	Change[b]	Ratio	Change[b]
Neg10[c]	1	0.89 ± 0.25	−11	0.88 ± 0.11	−12
Neg7.5	1.33	1.17 ± 0.07	−12	0.98 ± 0.23	−27
Neg5	2	1.86 ± 0.38	−7	1.39 ± 0.06	−31
Neg5p	2	2.04 ± 0.38	+4	1.82 ± 0.14	−9
Pos10	1	0.94 ± 0.03	−6	0.99 ± 0.01	−1

Liposomes of different composition were separated from free OPP (and from small size SUV) by ultracentrifugation (MLV) or by size exclusion chromatography (LUVET). The concentration of OPP and CH was determined by HPTLC as described in "Material and Methods". Each determination was performed in triplicate for two independent liposome formulations. Ratio values are expressed as mean ±SD.

[a] Initial ratio of OPP/CH that was used to prepare the liposomes.

[b] Percentage change of the ratio in comparison to the initial composition.

[c] For details of composition *see* Table 1.

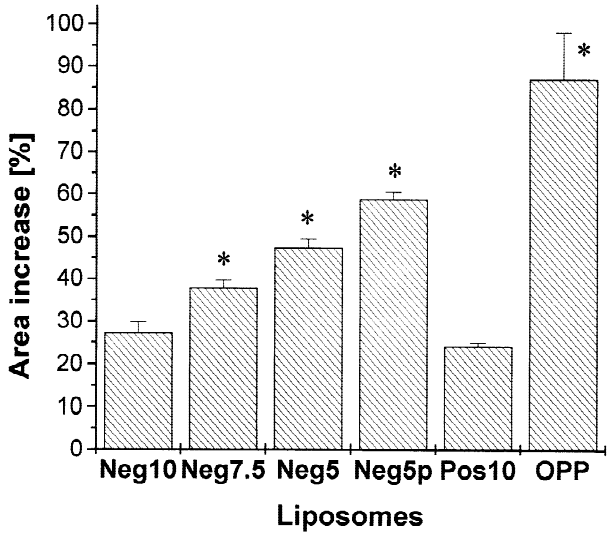

Fig. 3. Interaction of liposomal formulations of OPP liposomes with target monolayer. Different OPP liposomal preparations (for details of composition *see* Table 1) were injected into PBS below a lipid monolayer (POPC/POPE/POPS/CH 40:20:10:36; molar ratio) prepared in a commercially available Langmuir trough. The surface increase at a constant pressure was monitored as described in Materials and Methods. Data represent the change in surface area (mean ± SD of 3 independent experiments). *Indicates values significantly different from Neg10 ($P < 0.05$).

OPP was found to inhibit cell growth with an IC_{50} of 27 μM in the MT1 cell line and 45 μM in the SKBR-3 cell line, respectively (Table 3). The SKBR-3 cell line is obviously less sensitive to OPP than the MT1 cell line. All OPP-containing liposome formulations exhibited lower cytotoxicity than did OPP micelles. Cytotoxicity increased as the CH content decreased. This trend was less pronounced in the MT1 cell line. Negatively charged formulations containing equimolar amounts of cholesterol and OPP showed an almost 3-fold lower cytotoxicity compared to micellar OPP. Formulations containing positively charged lipids displayed higher cell toxicity than the corresponding formulations containing negatively charged lipids. PEG-lipid-containing systems exhibited the same cytotoxicity as liposomes of similar formulations but without sterical stabilization.

Table 3. Effect of liposomal composition on inhibition concentration (IC_{50}) for breast cancer cells

Cells	Composition[a]	IC_{50} [μM]	
	OPP/CH/X/PEG_{2000}DSPE[b]	Charge of the liposomes	
		Negative	Positive
MT1	10: 10:2:0	70.8 ± 7.8*	45.8 ± 4.5*
	10:7.5:2:0	48.8 ± 10.4*	36.4 ± 8.8
	10: 5:2:0	62.9 ± 6.0*	36.5 ± 6.7
	10: 5:2:1	52.8 ± 9.0*	30.8 ± 7.8
	Micellar OPP: 27.0 ± 8.2		
SKBR-3	10: 10:2:0	113.9 ± 42.9*	101.0 ± 13.3*
	10:7.5:2:0	80.1 ± 18.8*	52.6 ± 6.9
	10: 5:2:0	71.2 ± 19.6	84.9 ± 19.5*
	10: 5:2:1	48.9 ± 8.9	68.2 ± 5.5*
	Micellar OPP: 44.7 ± 6.7		

IC_{50} was determined by the MTT-assay after coincubation of appropriate concentrations with 1×10^5 cells/ml for 24 hours as described in Material and Methods. Given are the mean concentrations ± SD obtained from at least four independent experiments. Each determination was done in triplicate.

[a] Molar ratios of the components,

[b] with X = DCP for negatively charged and X = DDAB for positively charged liposomes

* Significantly different from micellar OPP; $P < 0.05$.

Discussion

Following up on our investigations of the biological effect of alkylphospholipid liposomes prepared from the

cancerostatically active phospholipid derivative OPP in vitro and in vivo (Arndt et al., 1999; Zeisig et al., 1998; Zeisig et al., 1996), biophysical model experiments were performed in this study to characterize in more detail the influence of the initial composition on the resulting properties of the formulation. The liposomal OPP formulations were characterized regarding their stability in terms of solute retention capacity, their interaction with a target lipid monolayer whose lipid composition resembles that of a tumor cell membrane (Punnonen et al., 1989) and the cytotoxic effect in vitro. Additionally, ^{31}P-NMR measurements and determination of the liposomal composition regarding OPP and cholesterol content were performed to obtain information on the composition-dependent extent of OPP incorporation into the liposomal membrane and the presence of free lipid in the liposomal suspension.

The results of this study show that the amount of OPP that can be stably incorporated in liposomes is dependent on composition, especially on the ratio between OPP and cholesterol. The majority of lipids were stabilized in a bilayer organization at an equimolar OPP-to-cholesterol ratio. With increasing OPP content the fraction of non-liposome-associated, micellar OPP increased. It comprised more than 30% of OPP in liposome formulations containing 58.8% OPP (Neg5 and Pos5 vesicles). The presence of an increasing micellar pool of lipids from which lipids can rapidly exchange into target membranes, was found to correlate with the observed increase in cell toxicity. The cytotoxicity of these formulations was also found to be dependent on the cell line. The following discussion will focus on the structural changes observed in these systems and on their implications for liposomal drug delivery.

Both, OPP as well as cholesterol, are non-bilayer forming lipids. However, in combination they can adopt a lamellar structure. This has previously been reported for mixtures of lysolipids, lysolipid analogues and detergents with cholesterol, DOPE or fatty acids (Jain et al., 1980; Madden & Cullis, 1982; Perkins et al., 1997; Ramsammy & Brockerhoff, 1982; Van Echteld et al., 1981) and can be rationalized in terms of their 'molecular shapes' (Cullis & de Kruijff, 1979; Israelachvili, Marcelja & Horn, 1980; Madden & Cullis, 1982). Lipids such as OPP and PEG-DSPE with a large headgroup area and a small hydrocarbon cross-sectional area have an inverted cone-like geometry. In isolation they self-assemble into micelles. On the other hand, lipids with a small headgroup area relative to the hydrocarbon chain area are cone-shaped and adopt "inverted" lipid phases such as the inverted hexagonal (H_{II}) phase formed by DOPE. Similarly, cholesterol has the shape of a truncated cone (Carnie, Israelachvili & Pailthorpe, 1979). Lipids that are cylindrical in shape, having nearly equal headgroup to hydrocarbon area, self-assemble into lipid bilayers. In combination shape-complementary lipids can adopt a cylindrical shape and assemble into a bilayer. From this it is clear that optimum bilayer stabilization will depend on the molar ratios of the lipids and the particular combination of the individual lipid species (Madden & Cullis, 1982). The OPP/cholesterol systems formed mostly bilayers at an equimolar OPP-to-cholesterol ratio. An increase in OPP content resulted in increased formation of micelles as well as a reduction in the number of the liposomes as evidenced by the decrease in trapped aqueous volume and the NMR results (Table 1, Figs. 1 and 2). These changes are consistent with the shape concept. In an excess over cholesterol the wedge-shaped OPP will destabilize the bilayer progressively, inducing the formation of structures exhibiting higher curvature such as micelles and small liposomes. About 85% of the phosphate groups were found in ^{31}P-NMR-measurements to be accessible to an externally added line-broadening agent in the formulation containing 58.8% OPP (Fig. 2). This is more than can be accounted for by the 30% fraction of micellar OPP determined from size exclusion chromatography (Table 2) and ^{31}P-NMR (Fig. 1). It implies an asymmetric distribution of OPP. This is in agreement with previous reports. For example, lyso-PC was found to localize preferably in the outer monolayer in lyso-PC/CH (1:1) SUVs with an outside-to-inside ratio of 6.5 (Kumar et al., 1988). Similar structural changes as described above were observed in PC/lyso-PC systems (Hauser, 1989). Incorporation of cholesterol in these systems was found to counteract the 'bilayer-destabilizing' role of lyso-PC (Van Echteld et al., 1981).

A similar effect was obtained after incorporation of PEG-DSPE, which seems to stabilize the membrane in a similar way and prevents the release of liposomal components. The covering of the liposomes by the highly hydrophilic polyethylene layer causes a fixed aqueous layer around the outer membrane (Zeisig et al., 1996), which may be responsible for the improvement of the membrane stability. The biological effects of these sterically stabilized vesicles were not affected because cytotoxicity (Table 3) and hemolysis (Zeisig et al., 1998) were similar to that of liposomes with a same composition but without sterical stabilization.

A further point of discussion concerns the stability of OPP liposomes in serum. Liposome formulations with higher OPP content exhibited increased serum stability (Table 1). This increase in stable encapsulation is not immediately obvious but may be related to membrane-packing properties. The same correlation between calcein release and OPP content was already observed in serum-free buffer, but on a lower level (Zeisig et al., 1998). Transient small holes in the liposomal bilayer, which allow calcein to diffuse out of the liposomal interior, may be responsible for the described leakage. The appearance of such transient holes is mainly dependent on the packing parameters and the flexibility of the mem-

brane. It could be further hypothesized that liposomes containing a larger amount of the wedge-shaped OPP and exhibiting larger curvature may be able to better compensate the effect of serum protein association with the membrane by filling up gaps created in the membrane. This explanation is substantiated by the observation that in POPC SUVs, lysolipids do not disrupt the bilayer to the same extent as in planar bilayers, but actually tighten it, by filling up gaps between the tilted POPC cylinders (Kumar et al., 1988). Measurements of the membrane fluidity are expected to contribute also to a better understanding of this correlation between composition and release of entrapped marker.

OPP, similar to lysolipids and lysolipid analogues, exhibits strong hemolytic activity in pure micellar form. Incorporation into a bilayer reduces this unwanted membrane-lytic side effects (Zeisig et al., 1998) but may also reduce overall biological activity. The toxicity of liposomal OPP formulations was strongly dependent on the amount of cholesterol in the formulation and as a result of this, in the amount of free OPP. It is known that alkylphospholipids and etherlipids interfere with membrane-associated processes in cells, especially with the signal transduction pathways (Ergezinger, Vehmeyer & Unger, 1999; Maly et al., 1995). This requires incorporation of these molecules into cell membranes and affords the presence of free or easily exchangeable lipid molecules and may explain the correlation between presence of micelles and cytotoxicity found in this study.

In summary, our findings demonstrate that the cytotoxicity of OPP-containing formulations is related to the amount of exchangeable, free or micellar OPP present in these formulations. Accordingly, cytotoxicity decreased with increasing cholesterol content and increased in the presence of PEG-DSPE. The sign of the surface charge had no effect.

This study was supported by the Deutsche Forschungs Gemeinschaft, grant AR 249/2 and, in part, by the Berliner Krebsgesellschaft. We gratefully acknowledge the technical assistance of Anne-Dorothe Teppke, Adnan Ibikli and Sabine Bucke.

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