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Biochimica et Biophysica Acta 1414 (1998) 238–248

BIOCHIMICA ET BIOPHYSICA ACTA
BBA

Physical properties and pharmacological activity in vitro and in vivo of optimised liposomes prepared from a new cancerostatic alkylphospholipid

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Received 16 July 1998; received in revised form 1 September 1998; accepted 7 September 1998

Abstract

Liposomes from octadecyl-(1,1-dimethyl-4-piperidino-4-yl)-phosphate (OPP), a new alkylphospholipid derivative with an improved cancerostatic activity, were prepared for the first time and the activity in vitro and in vivo was characterised. The formation of liposomes (MLV, SUV and LUVET) differing in cholesterol content, charge, and sterical stabilisation is possible without serious problems, despite the lysolipid-like structure of the OPP. Liposomes with a low amount of cholesterol and with PEG_{2000}DSPE-coating were the most stable OPP liposomes, both in buffer and in serum. The cytotoxicity of micellar or liposomal OPP against breast cancer cell lines in vitro was in the range of 20–60 μM. The cytotoxicity of the liposomal formulation was inversely related to the content of cholesterol, whereas the sterical stabilisation and/or the incorporation of a positive charge had only a very moderate modulating effect on the inhibition of cell proliferation. The strongest antitumour effect on the xenotransplanted breast cancer MT-3 in vivo was obtained with sterically stabilised OPP liposomes with low CH content. The beneficial therapeutic effect of these liposomes was accompanied by better tolerance and a significant inhibition of haemolysis compared to micellar OPP. © 1998 Elsevier Science B.V. All rights reserved.

Keywords: Liposome; Alkylphospholipid; Anticancer activity; Stability; Cationic liposome; Cytotoxicity

1. Introduction

Successful treatment of high-incidence breast cancer is still a serious problem. The search for new therapeutic strategies, or the improvement of 'classical' treatment approaches, therefore remains challenging. One promising new group of drugs without DNA interaction consists of ether- and alkylphospholipids, because the cell membrane is used as a target for therapeutic intervention. These compounds are especially effective against breast cancer in vitro [1,2] and in vivo [3,4], as well as for the clinical treatment of metastases [5].

Abbreviations: APC, alkylphosphocholine; CH, cholesterol; DCP, dicetylphosphate; DDAB, dimethyl-dioctadecylammonium bromide; HPC, hexadecylphosphocholine; IC_{50}, concentration of drug required to inhibit cell growth by 50%; LUVET, large unilamellar vesicle made by extrusion technique; MLV, multilamellar vesicles; OPP, octadecyl-(1,1-dimethyl-piperidino-4-yl)-phosphate; PBS, phosphate-buffered saline solution; PEG, polyethyleneglycol; PEG_{2000}DSPE, *N*-(*O*-methyl-polyethylenglycyl)-1,2-distearyl-*s*,*n*-glycero-3-phosphoethanol-amine; RES, reticuloendothelial system; SUV, small unilamellar vesicle

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PII: S0005-2736(98)00171-0

A serious, dose-limiting side effect of these lipids is haemolysis, which can be prevented if the lipids are used in liposomal form [6,7], although this is probably accompanied by a reduction in therapeutic activity, as has been shown at least in vitro [2].

A second advantage in the use of liposomal vesicles is the variability of preparation methods leading to liposomes with different pharmacological behaviour.

Based on our experiences with hexadecylphosphocholine and its analogues with various chain lengths, we wanted to investigate the newly developed octadecyl-(*N*,*N*-dimethyl-piperidino-4-yl)-phosphate (OPP, Perifosine, D21266, Fig. 1), which had recently been reported to have an improved activity against DMBA-induced mammary tumours of the rat [8].

The aim of this study was to compare the antitumour activity of OPP and HPC in a panel of four different human breast cancer cell lines. Furthermore, and more importantly, we were interested in finding liposomal preparations with cytostatic activity similar to micellar OPP, but with all the advantages of liposomes in vivo.

This study focused on the influence of the composition, especially with regard to cholesterol content, charge, size and sterical stabilisation on the physical properties and on the cytotoxic effect in vitro, as well as on the therapeutic activity in vivo.

2. Materials and methods

N-(*O*-Methyl-polyethylenglycyl)-1,2-distearyl-*s*,*n*-glycero-3-phosphoethanol-amine, Na-salt (PEG_{2000}-DSPE) with about 45 repetitive ethoxy-units were purchased from Sygena (Liestal, Switzerland). OPP and HPC were a generous gift from Dr. Hilgard (ASTA Medica, Frankfurt, Germany). Dicetylphosphate (DCP), cholesterol (CH) and calcein were purchased from Serva (Heidelberg, Germany). CH was recrystallised from acetone prior to use. Triton X-100 was a product of Ferak (Berlin, Germany). CH_2Cl_2 and MeOH from Merck (Darmstadt, Germany) were used in Lichrosolv gradient grade quality. PBS and foetal calf serum (FCS) are products of Gibco, Life Technologies (Eggersstein, Germany).

2.1. Liposome preparation and characterisation

MLV at a concentration of 10 mM were prepared by the lipid film/hydration method from appropriate mixtures of stock solutions of the components in CH_2Cl_2/MeOH, (7:3, v/v). The resuspension of the lipid film in PBS or calcein containing water (30 mM) resulted in plain or marker entrapping MLV. LUVET were prepared from these MLV as described previously [9] by repeated extrusion through polycarbonate filters (diameter of pores, 100 nm) using a LiposoFast Basic System (Avestin, Ottawa, Canada) until the suspensions were unimodal. In some cases, it was additionally necessary to sonicate the vesicles to obtain a unimodal liposomal population (see Table 1). Vesicle size determination was performed by dynamic light-scattering measurement with a Coulter Counter N4 MD model and the AccuComp System from Coulter Electronics (Hialeah, USA). Size is expressed in nm as unimodal mean diameter ± S.D. and size distribution as polydispersity index (PI) – varying from 0 (entirely monodisperse) to 1 (completely polydisperse suspension).

All the concentrations for liposomes reported here in this study are based on the concentration of the active OPP and were determined by HPTLC measurements.

2.2. Stability of liposomes

2.2.1. Buffer stability

Three batches of each liposome preparation with encapsulated calcein (30 mM) were prepared immediately after the separation of the liposomes from unencapsulated calcein by gel chromatography (Sephadex G50) and by diluting the liposomes to about 100 nmol/ml with PBS buffer. The suspensions were stored at 4°C until measurement.

At appropriate time points 100 μl/well of these liposome dilutions were applied to a 96-well microtitre plate (Maxisorp black, Nunc, Kamstrup Denmark) in triplicate. The amount of released calcein was directly determined by fluorescence measurements at F_{ex} 485 nm and F_{em} 510 nm with a SLT Fluostar plate reader (TECAN, Crailsheim, Germany) and calculated from a standard curve. The total amount of encapsulated calcein was determined

in a similar way after destruction of the liposomes by adding 50 μl of Triton X-100 to each well.

2.2.2. Serum stability

Two different liposome preparations were diluted with a 50% mixture of foetal calf serum/PBS to a concentration of 100 μM in duplicate and stored for different periods of time at 20°C. The solutions (each 100 μl) were measured for calcein quantification with the SLT Fluostar plate reader as described above.

2.3. Cell growth inhibition experiments in vitro

Human breast cancer cell lines MT-1 and MT-3 [10], MaTu [11], SKBR3 (ATCC, Parklawn, USA), all oestrogen receptor negative, were cultured in RPMI-1640 medium (Gibco) or McCoy's 5A medium (SKBR3) and supplemented with 2 μmol/ml L-glutamine (Flow Labs), penicillin G (100 U/ml), streptomycin (100 μg/ml, both from Gist-Brocades) and heat-inactivated foetal calf serum, (10% FCS, Gibco). All the reagents were free of endotoxin contaminations.

Cells in the exponential growth phase were seeded in a density of 1×10^5/ml in a 96-well microtitre plate (Falcon) on the day before the start of the incubation with the drug. Liposomal and free drug were serially diluted with medium to a concentration between 200 and 12.5 μM and given to the cells for 24 h in triplicate. Cells were then washed twice with PBS before fresh medium with MTT (0.5 mg/ml) was added for further 4 h of incubation. Finally, 190 μl of the supernatant was carefully removed, the formed formazan completely resolved in isopropanol-HCl, followed by 10 min of shaking. The quantification of the formazan was done by absorption measurements using a SLT-plate reader (TECAN, Crailsheim, Germany) at 530 nm. The percentage inhibition was calculated in relation to the growth of control cells handled similar, but without drug exposure.

The IC_{50} data were determined from the growth inhibition curves for each individual experiment and given as mean ± S.D. of at least four independent experiments.

2.4. In vivo experiments

Female NCR *nu/nu* mice, weighing 18–24 g, were used for the investigation. They were maintained under standardised (24°C, 50% humidity) sterile conditions, received sterile bedding and food (Sniff, Svest, Germany) and acidified drinking water.

All animal experiments were performed in accordance with the Animal Welfare and Ethics Rules licensed by the Senate of Berlin (Number G0090/94).

2.4.1. Therapeutic experiment

Pieces of the MT-3 human breast cancer with a size of 2–3 mm diameter were transplanted s.c into the left flank of eight anaesthetised mice/group. Treatment with micellar OPP or different OPP-LUVET (each 25 mg/kg/day) or saline (control) started when tumours were palpable (4–5 mm diameter) and was done i.p. on days 11–15 and 18–22 after tumour transplantation. Size of the tumours was measured twice/week with callipers. Tumour volume was calculated as follows: $\text{width}^2 \times \text{length}/2$. Median tumour volume is given as relative tumour volume (RTV) calculated for each measurement in relation to the starting tumour volume at the first treatment day. The percentage of RTV of treated to control group (T/C %) is used for characterisation of drug activity. Lethality and body weight change were determined as toxicity parameters.

2.4.2. Determination of the haemolytic activity in vivo

Female NCR *nu/nu* mice were injected i.p. with 100 mg/kg OPP in micellar or liposomal formulation (6 mice/group). After 1 h, blood was taken from the retro-orbital venous plexus. The obtained serum was diluted 1:5 with PBS and its extinction was measured at 540 nm. The extinction was correlated with the haemoglobin content in the serum.

2.5. Statistics

Statistical comparisons for in vitro data were performed with the unpaired Student's *t*-test (two populations) and with the Mann–Whitney non-parametric test for in vivo results. Differences were considered to be significant at $P < 0.05$.

3. Results

Liposomes were prepared for the first time from OPP and DCP (negatively charged) or DDAB (positively charged) at a ratio of 10:2, with a cholesterol content between 27.8 and 45.5% of the total lipids (this corresponds to a molar ratio of OPP/charged compound/CH between 10:2:5 and 10:2:10). In view of the therapeutic experiments in vivo, we additionally added PEG_{2000}DSPE (10 mol% of OPP) to some compositions to obtain sterically stabilised liposomes.

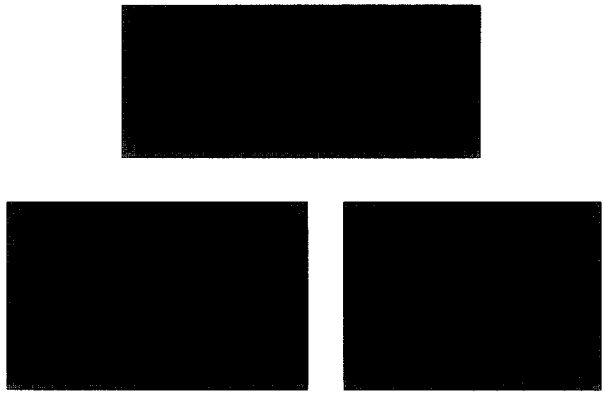


Fig. 1. Basic structure of alkylphospholipids.

3.1. Physical properties of OPP liposomes

Positively charged MLV were found to be more heterogeneous than negatively charged MLV and had a broader size distribution as indicated by the standard deviation and the PI (Table 1).

Small liposomes (LUVET) with a mean diameter between 100 and 200 nm were prepared from MLV by the extrusion technique. Whereas both 10:10 compositions (OPP/CH; Neg10, Pos10; for abbreviations and composition in detail see Table 1) were extruded without any problem, this method was increasingly hindered with decreasing CH content of the liposomes. MLV with a molar ratio of 10:5 (OPP/CH) could only be converted to small vesicles by sonication, and not by extrusion.

This difference between 10:10 LUVET (control liposomes) and vesicles with a lower CH content was additionally confirmed by the increased mean size, as well as by the higher polydispersity index of the liposomes. All of these preparations were more heterogeneous than the control liposomes.

The LUVET were investigated for their stability in aqueous systems at 5°C for 4 months (Fig. 2). The

Table 1
Characterization of OPP-liposomes

Type	Name	Composition (molar ratio)[a]	OPP/CH (% of total lipid)	Size (nm)[b]	PI[c]	Marker (mmol/mol)[d]
LUVET	Neg10[e]	10:10:2:0	45.5/45.5	103.0 ± 9.5	0.122	83.07 ± 4.41
	Neg7.5	10:7.5:2:0	51.3/38.5	135.0 ± 49.1	0.287	67.75 ± 3.73
	Neg5	10:5:2:0	58.8/29.4	125.4 ± 45.3	0.280	29.23 ± 1.18
	Neg5P	10:5:2:1	55.6/27.8	133.6 ± 46.1[g]	0.239	39.38 ± 4.59
	Pos10[f]	10:10:2:0	45.5/45.5	108.8 ± 28.4	0.097	86.87 ± 3.36
	Pos7.5	10:7.5:2:0	51.3/38.5	186.5 ± 77.5[g]	0.557	77.95 ± 13.7
	Pos5	10:5:2:0	58.8/29.4	104.4 ± 44.9[g]	0.176	20.91 ± 1.46
	Pos5P	10:5:2:1	55.6/27.8	126.5 ± 47.7	0.335	37.15 ± 2.09
MLV	Neg10	10:10:2:0	45.5/45.5	324.5 ± 114.2	0.737	n.d.
	Neg10P	10:10:2:1	43.5/43.5	620.6 ± 282.8	0.915	n.d.
	Pos10	10:10:2:0	45.5/45.5	473.5 ± 208.9	0.878	n.d.
	Pos10P	10:10:2:1	43.5/43.5	354.4 ± 149.7	0.629	n.d.

[a]Liposomal composition OPP-CH-*x*-PEG_{2000}DSPE where *x* is a charged component.
[b]Unimodal results, mean ± S.D. for three measurements.
[c]Polydispersity index, calculated from unimodal results of size determination by DLS spectroscopy.
[d]Encapsulated fluorescence marker calcain, given is the amount of calcein/liposomal OPP.
[e]Neg: charged component *x* is dicetylphosphate.
[f]Pos: charged component *x* is dioctadecyl-dimethylamine bromide (DDAB).
[g]Obtained after sonication.

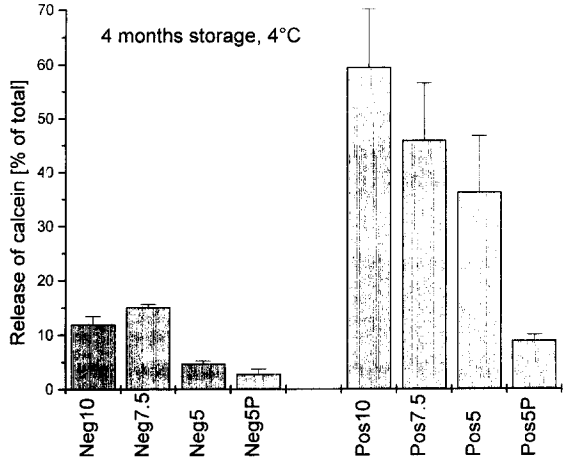


Fig. 2. Stability of OPP-liposomes after storage in buffer. Liposomes (about 100 μM) of different compositions (for details see Table 1) were stored in PBS buffer at 5°C for 4 months. Release of encapsulated calcein (absolute amounts are given in Table 1) was determined by fluorescence measurement at F_{ex} 495 nm/F_{em} 510 nm as described in Section 2. The mean release of marker for three different liposome samples compared to total amount is given in percent (±S.D.).

release of the fluorescence marker calcein was dependent on the charge and the cholesterol content as well. All positively charged liposomes released more marker than the negatively charged ones. The reduction of the amount of CH improved the stability, this was more pronounced for the positively charged liposomes than for the negatively charged ones. The three preparations with the highest release of more than 35% of encapsulated marker were Pos10, Pos7.5 and Pos5. All the other liposomes exhibited very similar stability, with a maximum release of only 10–15% (Fig. 2).

The stability of the liposomes in serum was investigated by incubation of calcein encapsulating vesicles in PBS/foetal calf serum (1/1, v/v) mixtures for at least 6 days, followed by fluorescence measurements of the released marker (Fig. 3). The same time dependency as described earlier [9] was found, with a fast release within the first minutes, followed by a plateau phase with only a marginal additional release and with a stronger final release after more than 2 days (Fig. 3A).

The liposomes with a 10:10 composition exhibited the highest absolute and percentage release. After about 50 h of incubation, for example (Fig. 3B), more than 2000 pmol/ml marker were released by Neg10 and Pos10 liposomes. This corresponds to 63.4 and 83.1% of total marker, respectively.

The reduction of cholesterol decreased the release of marker. The preparations with only 50% CH content were the most stable liposomes, with the release of an absolute amount smaller than 800 pmol/ml. The incorporation of PEG_{2000}DSPE resulted in additional stabilisation. Thus, Pos5P-liposomes released 39.5%, the Neg5P-liposomes only 32.9% of the total encapsulated marker (Fig. 3A,B). Comparing the data with other incubation times results in a similar gradation of the influence of composition, as we have demonstrated here for this long time incubation.

3.2. Cytotoxic effects in vitro

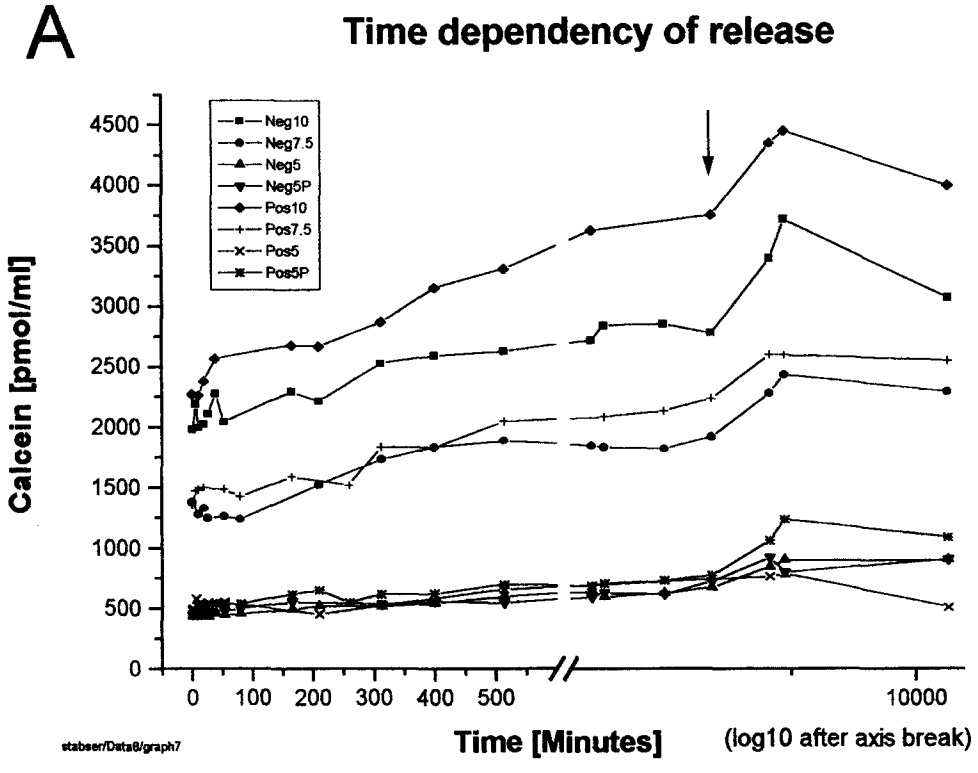
After summarizing all the data, we determined cytotoxicity, expressed as the concentration necessary to inhibit the cell growth to 50% (IC_{50}), to be in the range of 20–100 μM for an incubation of 10^5 cells/ml for 24 h (Fig. 4). Micellar OPP was significantly better than HPC in 3 of 4 cell lines. The IC_{50} data determined in the SKBR3 cell system were the highest for all investigated cell systems (Fig. 4).

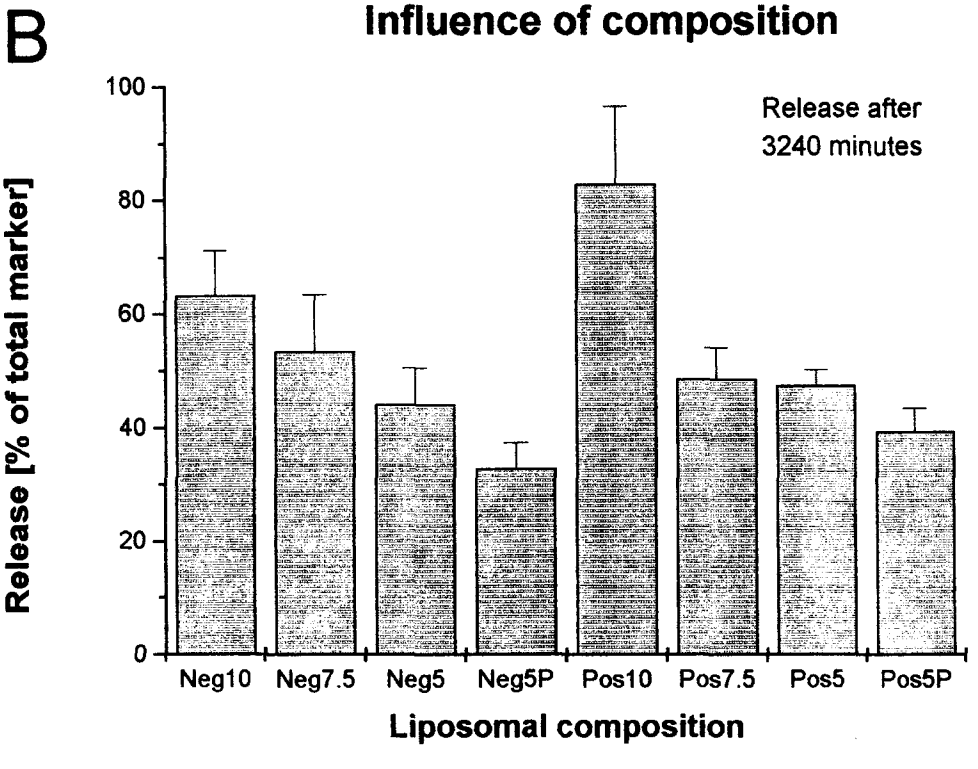
For MT1 and SKBR3 we found again the effect already known from HPC liposomes. The liposomal preparations with a high cholesterol content (Neg10, Pos10) were significantly less toxic ($P < 0.05$) to the cells than the micellar compound (Fig. 5). In contrast, MaTu and MT3 were found to be similarly sensitive to OPP and OPP liposomes, independent of the composition.

The reduction of CH increased the cytotoxic effect against all cell lines investigated in this study and resulted in lower IC_{50} data. In the MT1 and SKBR3 cell model, 6 of 12 liposomal preparations

→

Fig. 3. Stability of OPP-liposomes after incubation in serum. Two different liposome preparations of each composition were incubated in 50% foetal calf serum/PBS (100 μM). The samples were measured after appropriate times in duplicate as described in Fig. 2. The amount of released marker is given as mean of two measurements each done in duplicate. (A) Time course for the release of calcein. (B) Influence of composition on marker release, given in percent of total encapsulated calcain (mean±S.D.) after a storage of 54 h (indicated by the arrow in A).


A
Time dependency of release
Neg10
Neg7.5
Neg5
Neg5P
Pos10
Pos7.5
Pos5
Pos5P
Calcein [pmol/ml]
4500
4000
3500
3000
2500
2000
1500
1000
500
0
0
100
200
300
400
500
10000
stabser/Data8/graph7
Time [Minutes]
(log10 after axis break)


B
Influence of composition
Release after
3240 minutes
Release [% of total marker]
100
80
60
40
20
0
Neg10
Neg7.5
Neg5
Neg5P
Pos10
Pos7.5
Pos5
Pos5P
Liposomal composition

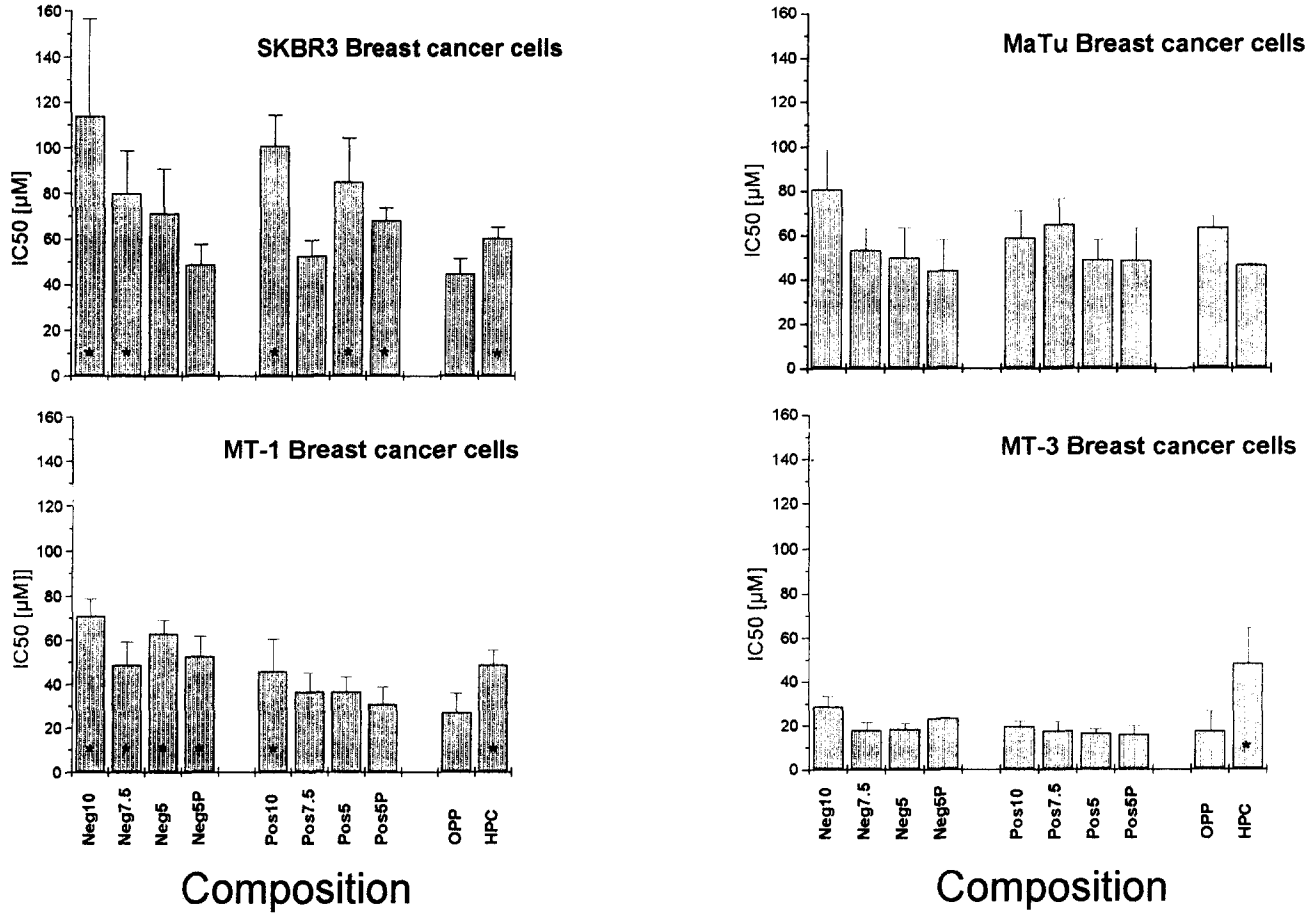


Fig. 4. Influence of liposomal composition on cytotoxicity in vitro. IC_{50} data for different breast cancer cell lines are given in part in all four panels. Cells were incubated with different liposomes for 24 h in triplicate as described in Section 2. Data given are the mean of four independent experiments. *Significantly different to OPP ($P < 0.05$).

with a reduced CH content were similarly as effective as OPP (Fig. 4, bars without *; $P > 0.05$).

The incorporation of PEG_{2000}DSPE for sterical stabilisation, or the use of DDAB as a charged component, had no or only a very weak influence on cytotoxicity as shown by comparing liposomes with similar CH content (Fig. 4).

A comparison of LUVET and MLV to determine the influence of liposome type showed the latter to be significantly less toxic ($P < 0.05$) to all the cell lines

Table 2
Pharmacological activity of OPP in micellar or liposomal formulation in breast carcinoma xenograft MT-3

Formulation	Dose (mg/kg/day)[a]	Toxic death/total number of mice	T/C[b] (%)	Body weight change (%)
OPP	25	0/8	66	−5
Neg10[c]	25	0/8	46	−4
Neg7.5	25	0/8	63	−7
Neg5	25	1/8	77	−7
Neg5P	25	0/8	23[d]	−3

[a]i.p., on days 11–15 and 18–22 after tumour transplantation.
[b]T/C: treated to control tumour size (on day 29 after tumour transplantation).
[c]For composition see Table 1.
[d]Significantly different to OPP ($P < 0.05$).

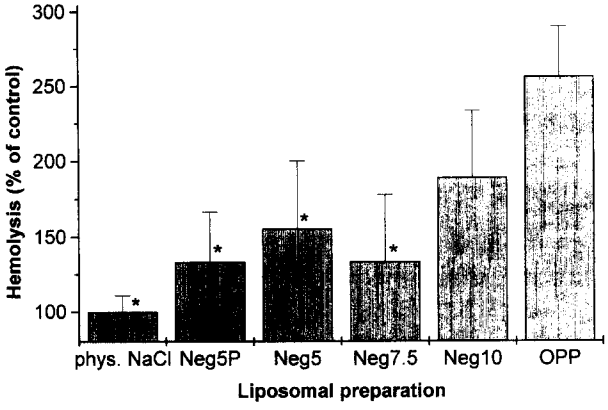


Fig. 5. Influence of liposomal composition on haemolysis in vivo. Female NCR *nu/nu* mice were injected i.p. with 100 mg/kg micellar or liposomal OPP. One hour later, the extinction of serum was measured at 540 nm as described in Section 2. The percentage increase in haemolysis compared to saline treated control group as mean ± S.D. of six mice is given. *Significantly different to micellar OPP ($P > 0.05$).

investigated. The MLV were also significantly less cytotoxic than the micellar OPP ($P < 0.001$; data not shown).

3.3. Therapeutic experiments in vivo

OPP in micellar and liposomal formulations was tested in an oestrogen receptor negative breast xenograft (MT-3). This model was chosen because of the remarkable antitumour activity of APL, especially in breast carcinomas [6,8,10]. In the experiment presented in Table 2, liposomes with different cholesterol content and sterical stabilisation were compared to micellar OPP. Clearly, the PEG-containing liposomes showing the highest stability and the lowest IC_{50} in vitro, exerted the most pronounced inhibition of tumour growth in vivo. The other liposomal preparations were, like OPP, only moderately active in this experiment, which was due to the relatively low dose used (25 mg/kg). Both lethality and body weight were unaffected or only marginally changed, respectively.

Fig. 5 presents the extinction of serum 1 h after administration of OPP in micellar or different liposomal formulations as an indication of the haemolytic potential of the amphiphilic compound. Clearly, micellar OPP and Neg10 liposomes led to a distinct induction of haemolysis, while all other preparations only marginally increased the haemoglobin content in the serum.

4. Discussion

Hilgard et al. [8] recently described a new alkylphospholipid derivative. These lipids are well known for their antitumour activity against a number of tumours in vitro and in vivo [1,12], and especially against oestrogen receptor negative mammary carcinomas [3,6]. In the new lipid OPP, the choline head group is substituted by the cyclic aliphatic piperidyl residue (Fig. 1). The marginal modification in structure changed the sterical properties slightly and led to an enhancement of the positive charge at nitrogen, compared to choline derivatives. This change resulted in a clear increase in the cytotoxic activity in vitro as compared to HPC, the main representative of the APL family, as demonstrated in this study and by Hilgard et al. [8].

The application of HPC and other APL in micellar form in vivo is accompanied by serious side effects, like haemolysis and gastrointestinal toxicity [4,13]. Haemolysis was also observed in our experiment using micellar OPP. The use of OPP liposomes instead of the micellar alkylphospholipid reduced both side effects as demonstrated earlier for HPC liposomes [4,7].

The aim of this study was, then, to characterise liposomes prepared for the first time from this new APL and to optimise the liposomal formulation with regard to stability and pharmacological properties. Liposomes prepared so far from HPC were found to be less cytotoxic in vitro than the micellar solution of similar concentration [2].

OPP-MLV and vesicles of a defined, small size (LUVET with a diameter < 200 nm) with different amounts of CH, with a negative or positive charge, and in some cases with sterical stabilisation by PEG coating, were prepared and investigated for the first time.

The liposomes were first characterised for their physical properties. The vesicle formation was very similar to that seen for alkylphosphocholine liposomes. But in contrast to our former impression [2], that a molar ratio of 1:1 for alkylphosphocholine/CH seems to be necessary to obtain stable liposomes, it was found in the present study that a reduction of the CH content to 50 mol% is possible with still continued vesicle formation.

There was no problem with negatively charged lip-

osomes, even after the addition of $PEG_{2000}DSPE$, one of the most frequently used compounds to achieve sterical stabilisation [14], as could be shown by size characterisation as well as for stability measurements in an aqueous system and in serum. If the charged component DCP was substituted by the positive compound DDAB, the situation became more complicated. The aggregation sometimes seen after MLV preparation and the decreased stability in buffer clearly indicated a stronger disorder of the membrane than found for negatively charged vesicles. It was demonstrated recently [15] that the incorporation of more than 3 mol% of a single positively charged compound resulted in a more fluid liposomal membrane. This probably explains the low stability of the positively charged liposomes in buffer compared to the negatively charged ones, because the amount of DDAB used was always higher than 8.7 mol%.

The situation in serum was different from that in PBS. Vesicles with similar composition, but different charge, exhibited similar stability. The release was only related to the content of CH in the membrane.

Interestingly, the stability based on the release data was found to increase with decreasing CH content. Usually, the contrary would be expected because the decrease in CH is accompanied with a reduction of membrane fluidity [16,17]. We assume that the single chain lipid OPP requires an optimum amount of cholesterol to stabilise the membrane maximally, which seems to be achieved better with a ratio of 2:1 than 1:1 for OPP/CH.

Summarizing these data, the best liposomal preparations with regard to physical properties were found to be the liposomes Neg5, Neg5P or Pos5P.

In the next step, we were interested in the effects of the different preparations against tumour cells in vitro. The cells used in our experiment showed different sensitivity to micellar and liposomal OPP, indicating different mechanisms for the binding or uptake.

We first investigated the effect of cholesterol on cytotoxicity in vitro. It is known that cholesterol in liposomal preparations sometimes have a diminishing effect on the cancerostatic activity when liposomes are used as carriers for classical anticancer drugs [18,19]. Our data demonstrated that the incorporation of a high amount of CH reduced the cytotoxicity compared to micellar OPP for all the investigated cell lines. In contrast, the reduction of liposomal cholesterol correlates with an increase of the cytotoxic effect in vitro. This could be explained by the increase in membrane fluidity caused by CH reduction [17,20], which probably facilitates (specific) interactions between components of the liposomal membrane with those of the cellular membrane. But these findings are only partially consistent with the in vivo results described previously [20], which demonstrated that the uptake of liposomes by tumour cells is not dependent on the content of CH and thus on the fluidity of the membrane, but is related to the size of liposomes. Size dependency explains, on the other hand, the decreased cytotoxic activity found for the large OPP-MLV, most likely by an inhibition of the trans-bilayer transport of the MLV into the cells.

The increase in cytotoxicity with decreasing liposomal CH could also be the result of a release of micellar OPP from the vesicles or the destruction of the liposomes. But both the differences in cell sensitivity to micellar and liposomal OPP found for different cell lines, and the high stability in serum of OPP-LUVET with a lower amount of CH, made such an explanation relatively unlikely. Nevertheless, more experiments are necessary to explain these observations.

The incorporation of the positively charged compound DDAB, instead of DCP, had no modulating effect on the inhibition of growth in the cells investigated. DDAB seems to be well-tolerated by the cells used.

It still remains unclear why sterically stabilised liposomes (Neg5P and Pos5P) showed similar in vitro activity as liposomes of the same composition, but without pegylation (Neg5 and Pos5). Taking into account that there is a specific uptake by the cells, and that the hydrophilic chains of the PEG lipids cover the liposomes, the more or less specific liposome–cell interaction should be reduced for the pegylated liposomes, similar to the situation demonstrated recently for macrophages ([9,21], for review see [14]). This indicates a different mechanism for liposome uptake by macrophages and breast tumour cells, as has also been discussed when comparing CV1 green monkey kidney cells with J774 macrophage-like cells [22]. To our knowledge, it is not

yet known whether the sterical stabilisation generally affects liposome–cell interaction or if a specific prevention of liposome recognition and uptake only by macrophages is obtained.

Based on the in vitro results, small, sterically stabilised liposomes with a reduced content of CH should be the most active liposomal formulation in vivo. This could be confirmed in the animal experiments. OPP induced only a moderate, and not significant, inhibition of tumour growth in a human xenograft breast cancer model at the relatively low and tolerable dose of 25 mg/kg/day administered i.p. The intraperitoneal route of administration chosen for these experiments resulted in better tolerance of the drugs used compared to the i.v. route and prevented the destruction of the liposomes after oral application. No side effects were observed in the peritoneum.

The antitumour effect could be improved remarkably when OPP was administered in a stable PEG-containing liposomal formulation with a reduced CH content. In addition, the haemolytic potential of alkylphospholipids demonstrated with micellar OPP could be prevented if OPP was applied in liposomal form. The best tolerance was found for Neg5P-liposomes.

It could be shown in this study that an improvement of the therapeutic index of the new alkylphospholipid OPP is possible if it is used in liposomal form. The careful selection of the properties of these OPP liposomes (especially concerning composition and size) increases the stability as well as the activity in vitro. The human MT-3 xenotransplanted breast cancer model revealed that the best liposomal formulation found in vitro (Neg5P) was also the best preparation in vivo with regard to therapeutic activity and the reduction of haemolysis.

OPP, even in liposomal form, is a powerful new alkylphospholipid derivative and the results of the first ongoing clinical trials with micellar OPP are awaited with great anticipation. Independent of this, further improvement of the OPP should be possible, especially in order to reduce side effects, if liposomal OPP, instead of the micellar formulations, is used for further clinical applications.

Acknowledgements

This work was supported by the German Research Foundation (Ar 249/1-2) and the Berlin Society of Cancer Research. The technical skills of A. Teppke, A. Ibikli, M. Lemm and M. Becker as well as the support by G. Hayes, S.Z. Kim, F. Freudenberg and I. Prinz are gratefully acknowledged.

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