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Antitumor Activity of Doxorubicin Encapsulated in Hexadecylphosphocholine (HePC) Liposomes against Human Xenografts on *Scid* Mice

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Abstract. *Doxorubicin was encapsulated into liposomes composed of hexadecylphosphocholine:egg yolk phosphatidylcholine:stearylamine (HePC:EPC:SA) 10:10:0.1 (molar ratio) (1) and EPC:SA 10:0.1 (molar ratio) (2). Liposomal formulations **1** and **2**, as well as free doxorubicin and free HePC, were tested in vitro against HCT116 human colon cancer cell lines and peripheral blood mononuclear cells (PBMCs) obtained from healthy donors, using the sulphorodamine B assay. The activity of doxorubicin was retained or slightly improved when entrapped into liposomes **1** and **2**, while liposomal formulation **1** incorporating doxorubicin was found to be less toxic against normal cells. The liposomes were tested in vivo against human colon cancer xenografts in scid mice. The antitumor activities of liposomes **1** and **2** were statistically similar to that of free doxorubicin, but their toxicity was significantly lower. Based on these results, the combination of HePC and doxorubicin in one liposomal formulation may be justified for further evaluation.*

Liposomes are considered to be a valuable tool in the fight against cancer as they can increase the therapeutic usefulness of the encapsulated anticancer drugs (1, 2). Liposomal doxorubicin is an example, since the high cardiotoxicity of doxorubicin limits its clinical use (3), despite a good anticancer activity against a variety of tumors (4). Liposomal doxorubicin, which is in clinical use in the USA and in Europe (5, 6), has proven to be active against many types of cancer, reducing the toxicity of the doxorubicin. Several clinical trials are in progress in order to evaluate the use of liposomal doxorubicin formulations, either alone or in combination with other anticancer drugs (7, 8).

Antitumor ether lipids are a relatively new class of anticancer agents. Their main cellular target is the plasma membrane and they do not interact directly with the nuclear DNA; their activities include induction of cell differentiation of leukemic cells, inhibition of angiogenesis and inhibition of tumor cell invasion (9). Antitumor ether lipids are divided into two subclasses: alkyllysophospholipids and alkylphosphocholines. The main representative of the alkylphosphocholines is hexadecylphosphocholine (HePC, miltefosine). This ether lipid is used clinically against breast cancer skin metastases in several European countries. It has been reported to inhibit the activity of protein kinase C and to depress the formation of inositol 1.4.5-triphosphate, while the concomitant mobilization of intracellular Ca^{+2} (10) HePC induces apoptosis (11) and interferes with membrane lipids (12).

Earlier efforts to prepare HePC liposomes were based on the observation that HePC and structurally-related alkylphosphocholines can form stable liposomes (13). Attempts to co-administer DNA-damaging agents (such as doxorubicin) or antimetabolites with ether lipids have also been reported previously (14, 15).

In this study, the *in vitro* and *in vivo* results obtained with two liposomal formulations free of cholesterol, encapsulating doxorubicin, composed of hexadecyl phosphocholine: egg yolk phosphatidylcholine: stearylamine (HePC:EPC:SA) 10:10:0.1 (molar ratio) (1) and egg yolk phosphatidylcholine: stearylamine (EPC:SA) 10:0.1 (molar

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ratio) (2), against the HCT116 cancer cell line and human colon cancer xenografts, are presented.

Materials and Methods

Materials. Hexadecyl phosphocholine (HePC) was a generous gift from Asta Medica/Baxter (Bielefeld, Germany). Egg yolk phosphatidylcholine (EPC) was purchased from Avanti Polar Lipids (AL, USA). Doxorubicin hydrochloride was purchased from Pharmacia (NJ, USA). Ammonium sulphate, TES (*N*-tris (hydroxymethyl) methyl-2-aminoethanesulfonic acid), Tris (tris (hydroxymethyl) aminomethane), stearylamine (SA), Sephadex G75, sulphorodamine B (SRB) and RPMI 1640 culture medium were purchased from Sigma-Aldrich (St.Louis, MO, USA).

Liposome preparation, doxorubicin encapsulation and determination of lipids and doxorubicin. Liposomes **1** and **2** were prepared using the reverse phase evaporation method (16, 17). The concentration of doxorubicin encapsulated into the liposomal formulations was measured on a Perkin Elmer UV-vis spectrometer at λ=481 nm after adding absolute ethanol. Prior to determination of the encapsulated doxorubicin, the non-encapsulated drug was removed by passing the samples through a Sephadex G75 column. HePC, EPC and SA were determined by high performance thin-layer chromatography coupled with a flame ionization detector (HPTLC-FID, Iatroscan MK-5, Iatron Lab. Inc., Tokyo, Japan) (18).

The liposome size and ζ-potential measurements were made at 25˚C and at an angle of 90˚ in a photon correlation spectrometer (Zetasizer 3000, Malvern, UK) and analyzed by the CONTIN method (MALVERN software). The physical stability of the liposomes at room temperature or at 4˚C for several weeks was studied by measuring their size and ζ-potential using photon correlation spectroscopy. Their chemical stability was assessed by monitoring the formation of conjugated dienes using UV spectrometry at λ=233 nm (19).

In vitro cytotoxicity studies. The cytotoxic activity of HePC as a solution (f-HePC) and as liposome (l-HePC; liposomal formulation **1** free of doxorubicin), as well as of free doxorubicin (f-doxorubicin) encapsulated into HePC liposomes (l-HePC/doxorubicin; liposomal formulation **1** encapsulating doxorubicin) or into liposomes without HePC (l-doxorubicin; liposomal formulation **2** encapsulating doxorubicin) were investigated against the HCT116 colon cancer cell line. Normal human peripheral blood monocytic cells (PBMCs), isolated from healthy human donors resting or activated with PHA (10 μg/ml), were tested. The HCT116 cells and PBMCs were grown in RPMI-1640 medium supplemented with 5% heat-inactivated fetal calf serum (FCS), 2 mM L-glutamine and antibiotics in a 37˚C humidified incubator and 5% CO_2 atmosphere. Cell viability was assessed by the trypan blue dye exclusion method at the beginning of each experiment and was always greater than 98%. The cells were then seeded into 96-well plates (Corning) in 100 μL of cell suspension/well and at a density of 5,000 cells, while PBMCs were seeded at a density of 150,000 cells/well. Subsequently, the plates were incubated for 24 h to allow attachment of cells prior to addition of the test agents. At the beginning of each experiment, a plate of each cell line was fixed *in situ* with TCA and SRB staining was performed to determine the cell population for each cell line at the time of drug addition (Tz).

HePC free or in liposomes and / or doxorubicin in solution or in liposomes were added to the culture medium at the end of the 24-h attachment period and at decreasing concentrations *i.e.,* at five consecutive 10-fold dilutions ranging from 0.01 to 100 μM of HePC for f-HePC and l-HePC or from 0.02 to 200 of doxorubicin for the f-doxorubicin and l-doxorubicin formulations. The cells were then incubated for an additional period of 48 h, under the same conditions as before the addition of the agents. Cultures where a corresponding volume of 100 mM TES, 100 mM NaCl (pH=7.5) buffer instead of the drugs/formulations was added served as negative controls. At the end of the incubation period, 50 μl ice-cold TCA (50% w/v) was added. SRB staining was then performed (20) and absorbance was measured at 530 nm on an EL-311 BIOTEK microelisa reader.

The parameters GI_{50}, TGI and LC_{50} were determined. Briefly, GI_{50} indicates the growth inhibition strength of the test compound and is calculated by the equation: $100 \times (T-T_z)/(C-T_z) = 50$. TGI indicates the cytostatic effect of the compound and is calculated by the equation: $100 \times (T-T_z)/(C-T_z) = 0$. LC_{50} indicates the compound concentration that induces cytotoxicity in 50% of the cells and is calculated by the equation: $100 \times (T-T_0)/T_0 = -50$. T and T_z are the absorbance values at the time when the test compounds are added (T_z) and after a period of treatment (T); C indicates the absorbance value measured in untreated cells (control) after an incubation period equal to the treatment period (21). Finally, in order to evaluate the efficacy of the different forms of doxorubicin in terms of safety, the therapeutic index (*TI*) was defined as the quotient of the parameter obtained with normal PBMCs divided by the mean of the corresponding parameter obtained with the cancer cells *i.e.* for GI_{50} $TI = GI_{50}^{PBMCs}/GI_{50}^{HCT\text{-}116}$, where GI_{50}^{PBMCs} is the GI_{50} parameter calculated for the PBMCs and $GI_{50}^{HCT\text{-}116}$ is the GI_{50} parameter calculated for HCT116 cells.

Animals. Thirty-one male 6-week-old NOD.CB17 $Prkdc^{scid}$/J mice (Jackson Laboratories/Charles River Laboratories, France), weighing 20–24 g, were housed under defined conditions with the temperature being maintained at 22±2 ˚C and the light controlled at a 12-h light and 12-h dark cycle.

Xenograft model development. HCT116 cells, which were grown for the *in vitro* studies, were implanted *s.c.* bilaterally into the rear axillary region of the recipient *scid* mice ($1x10^6$ cells/injection, 2 injections/mouse). When the tumor was measurable (0.06 g or 100 mm^3), the animals were divided randomly into 6 groups consisting of at least 5 mice each (day 13) and treatments started.

All formulations (Table I) were administered *i.p.* in a volume of 15 ml/kg of body weight. The doxorubicin was administered at a dose of 1mg/kg of body weight. The corresponding doses per kg of body weight of HePC and EPC were 0.4 mg and 0.8 mg, respectively. The applied schedule was 3 cycles of one injection daily for 5 consecutive days per week until the end of the experiment.

The length (a) and width (b) of the solid tumor mass were measured by caliper twice weekly, and the tumor volume (TV) was calculated as: $TV = (\alpha \times b^2)/2$, assuming unit density. $\Delta T/\Delta C$ % was determined by calculating the difference in the tumor volume of the treated group and dividing it by the difference in tumor volume of the control group, *i.e.* $\Delta T/\Delta C\% = 100x$ (mean ΔT of treated group)/(mean ΔC of control group), where $\Delta T = T-T_0$ and $\Delta C = C-C0$ (T0 and C0 is the average tumor weight at the beginning of the

Table I. *Composition of liposomal formulations (molar ratio), drug to lipid molar ratio and free drugs tested against HCT116 cell line (in vitro) and xenograft (in vivo).*

	Composition	Molar ratio	Doxorubicin/ phospholipids molar ratio
f-doxorubicin[a]	Doxorubicin[b]	-	-
l-doxorubicin[c]	EPC:SA	10:0.1	0.96 ± 0.05
l-HePC/doxorubicin[d]	HePC:EPC:SA	10:10:0.1	0.85 ± 0.04
l-HePC[e]	HePC:EPC:SA	10:10:0.1	-
f-HePC[f]	HePC[g]	-	-

[a]f-doxorubicin: free doxorubicin; [b]doxorubicin in solution; [c]l-doxorubicin: liposomal formulation **2**, encapsulating doxorubicin; [d]l-HePC/ doxorubicin: liposomal formulation **1**, encapsulating doxorubicin; [e]l-HePC: liposomal formulation **1** free of doxorubicin; [f]f-HePC: free HePC; [g]HePC: HePC in solution.

Table IIA. *GI_{50}, TGI, LC_{50} values of f-doxorubicin l-doxorubicin and l-HePC/doxorubicin against human colon cancer cell lines and PHA-activated peripheral blood mononuclear cells (PBMCs). The results are the mean of three independent experiments, SD<10%. Concentrations are expressed in μM*

	f-doxorubicin[a]			l-doxorubicin[b]			l-HePC/doxorubicin[c]		
Cell line	GI_{50}	TGI	LC_{50}	GI_{50}	TGI	LC_{50}	GI_{50}	TGI	LC_{50}
HCT116	0.31	2.56	8.54	1.21	21.32	127.2	0.14	1.07	4.69
PBMCs	17.1	45.7	74.22	NT[d]	NT	NT	35.46	151.58	229.0
TI[e]	55.16	17.85	8.69	-	-	-	253.29	141.66	48.83

[a]free doxorubicin in solution; [b]liposomal formulation **2**, encapsulating doxorubicin; [c]liposomal formulation **1**, encapsulating doxorubicin; [d]not tested; [e]therapeutic index (see text).

Table IIB. *GI_{50}, TGI and LC_{50} values of f-HePC and l-HePC against human cancer cell lines and PHA-activated peripheral blood mononuclear cells (PBMCs). The results are the mean of three independent experiments, SD<10%. Concentrations are expressed in μM.*

		f-HePC[a]			l-HePC[b]		
Origin of tumor	Cell line	GI_{50}	TGI	LC_{50}	GI_{50}	TGI	LC_{50}
Colon	HCT116	32.09	100.0	100.0	41.41	69.02	85.64
PBMCs		69.07	100.0	100.0	54.02	77.88	100.0
TI[d]		2.15	1.00	1.00	1.30	1.13	1.17

[a]HePC in solution; [b]liposomal formulation **1**, free of doxorubicin; [d]therapeutic index (see text).

treatment, T and C are the average tumor weights at a specified day for treated and control (untreated) tumors, respectively). The minimum level for activity is considered to be ΔT/ΔC% <42%, according to the National Cancer Institute standards.

The data collected included the number of animals that did not develop a tumor, the number of drug-related deaths of the mice and, finally, the number of days required for the tumor to reach a defined weight. The mouse body weight was monitored on the same day as the tumor measurement and was taken as a measure of side-effects, together with observation about the animals' behavior.

All animals were treated according to Greek laws (2015/92) and the instructions of the EU and the European Council (86/609 and ETS123, respectively) governing the use and handling of animals in experiments.

Statistical analysis. Statistical analysis of the effect of the formulations on tumor growth delay was performed using the one-way ANOVA, where that was applicable. In these cases, the means of the individual groups were compared *post hoc* using Tukey's HSD test. Where ANOVA criteria were not fulfilled, the Kruskal-Wallis test was used followed by the Games-Howell *post hoc* analysis, using SPSS for Windows release 11.0.0 (SPSS Inc., USA).

Results

The encapsulation of doxorubicin, stability studies, the physicochemical characteristics of the liposomal formulations and the release properties of the encapsulated doxorubicin, have been recently published (17).

The liposomal suspensions **1** and **2** were freeze-dried and resuspended in water in order to evaluate their size ζ-potential and doxorubicin encapsulation after reconstitution. The average size of the reconstituted liposomes **1** and **2** was 487.7±30.6 nm and 420.4±54.6 nm and their ζ-potential was –7.0±1.6 mV and –33.7±0.5 mV, respectively. The doxorubicin encapsulated in liposomes **1** and **2** was retained at a percentage of 99.62±5.4% and 97.0±2.6%, respectively, after their reconstitution. Furthermore, less than 1% of the lipids of the lyophilized liposomes **1** and **2** were oxidized during a period of 17 weeks of storage at 4˚C .

The release of doxorubicin from the liposome **1** in TES 100 mM NaCl 100 mM buffer (pH 7.5), and in RPMI-liposome formulation 1/1 v/v cell culture medium at 25˚C and at 37˚C was determined; 70.5% of doxorubicin was retained within the liposomes at 25˚C and 35.5 % at 37˚C in TES buffer after 24 h; 24.5% of doxorubicin was retained within the liposomes at 37˚C during the first 5 h, while 32.5 % of doxorubicin was retained within the liposomes at 25˚C after 24 h, in 50% RPMI cell culture medium for liposomal formulation **1**.

In vitro cytotoxic studies on human cancer cell lines and PBMC. The *in vitro* cytotoxic activity of the liposomal formulation **1** encapsulating doxorubicin

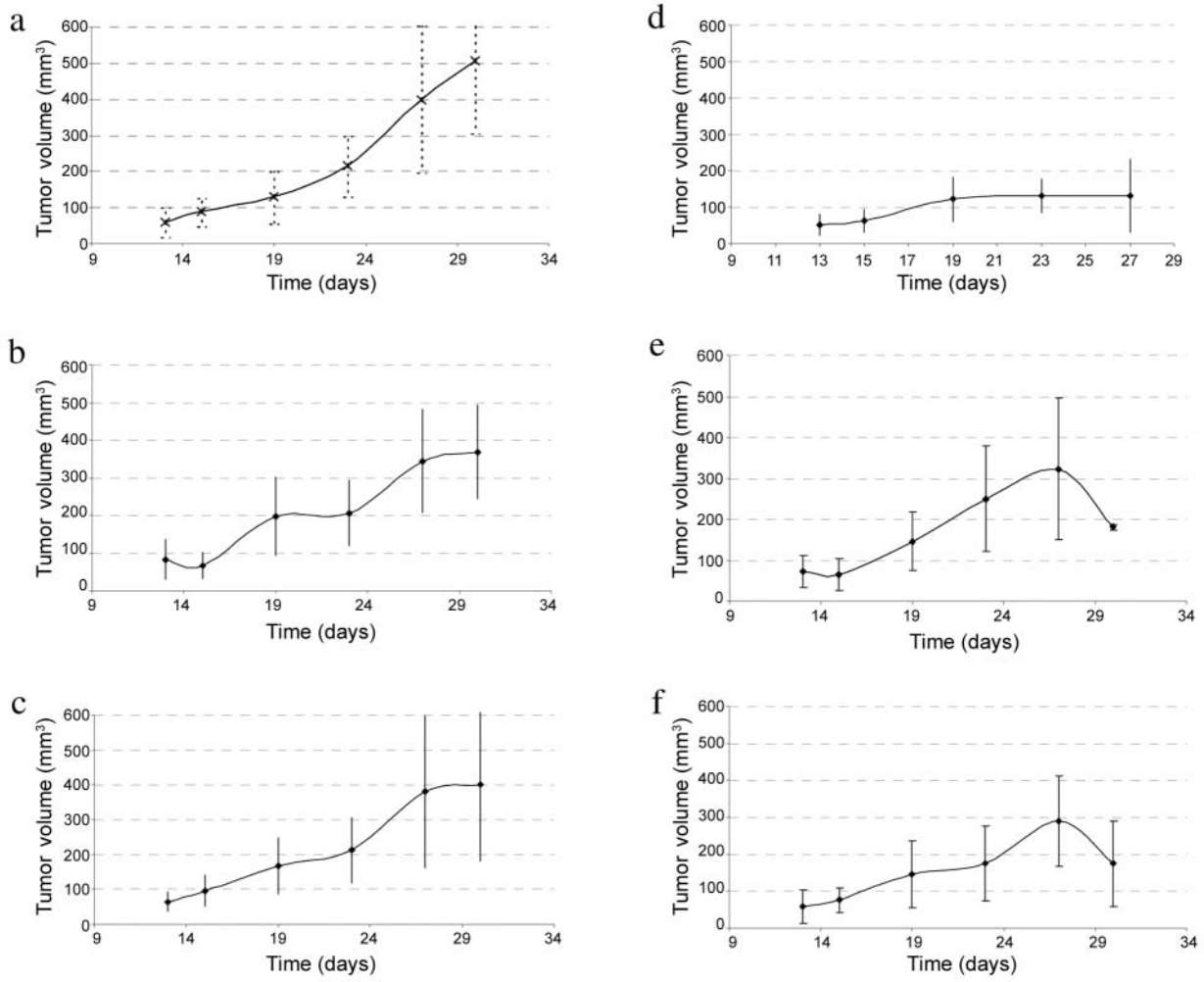

Figure 1. *The effect of the various treatments on the growth of HCT116 tumor inoculated into the two rear flanks of NOD CB17 Pr kdc^{scid} mice, as described in the text (see Materials and Methods). When the tumors were measurable (0.06 g or 100 mm^3, one group of mice randomly selected was left without receiving any further treatment and served as control (a)). The rest of the mice were randomly divided into 4 groups and received treatments with f-HePC (b); l-HePC (c); f-doxorubicin (d); l-doxorubicin (e); l-HePC/doxorubicin (f). Values are the mean ± SD (n=5) of one representative experiment. Numbers and arrows indicate the numbers of dead mice and the experimental day that the deaths occurred.*

(l-HePC/doxorubicin), was tested against the HCT116 cell line and PBMCs. For comparison, free HePC (f-HePC), HePC in liposomes without doxorubicin (l-HePC;liposomal formulation **1** free of doxorubicin), free doxorubicin (f-doxorubicin) and doxorubicin in liposomes without HePC (l-doxorubicin; liposomal formulation **2** encapsulating doxorubicin) were tested in parallel (Table IIA). f-Doxorubicin was highly active, exhibiting a GI_{50} at 2.56 μM, while encapsulation of doxorubicin into the liposomal formulation **2** (*i.e.* l-doxorubicin) reduced its activity, while its growth inhibiting activity was highly affected (0.31 μM for f-doxorubicin compared to 1.21 μM for l-doxorubicin, Table IIA). When both HePC and doxorubicin were incorporated into liposomes (*i.e.* l-HePC/doxorubicin; liposomal formulation **1** encapsulating doxorubicin), the activity of doxorubicin was increased and was comparable to the activity of the free drug, as far as the growth inhibiting activity of doxorubicin is concerned (GI_{50} 0.14 μM for liposomal formulation **1**). The results of the activity of l-doxorubicin against activated PBMCs showed reduced toxicity, although this liposomal formulation retained the activity of doxorubicin against HCT116 cancer cells. It is notable that l-HePC/doxo (*i.e.* liposomal formulation **1** encapsulating doxorubicin) although it showed improved activity against the HCT116 cells, was much less toxic against activated PBMCs. This resulted in a *TI* much higher for the l-HePC/doxo liposomal formulation **1** (253.29 compared to 55.16 for the f-doxorubicin, Table IIA)

Table III. *Relative tumor growth rate (ΔT/ΔC%) of the tested animal group.*

Experimental day[a]	f-Doxo[b]	l-Doxo[c]	l-HePC/ Doxo[d]	l-HePC[e]	f-HePC[f]
15	39.43	–24.95	64.20	113.91	–60.70
19	100.50	106.44	126.74	148.64	163.92
23	51.41	114.53	75.10	95.11	79.32
27	23.53	73.45	67.84	92.69	76.84
30	-	27.0	24.36	74.81	63.71

[a]day after inoculation of the mice with human colon cancer cells; [b]free doxorubicin in solution; [c]liposomal formulation **2**, encapsulating doxorubicin; [d]liposomal formulation **1**, encapsulating doxorubicin; [e]liposomal formulation **1**, free of doxorubicin; [f]HePC in solution.

Table IIB shows that incorporation of HePC into liposomes did not alter its *in vitro* activity. HePC incorporated in HePC:EPC:SA 10:10:0.1 liposomes (*i.e.* l-HePC, liposomal formulation **1**, free of doxorubicin) retained their activity at the same level as f-HePC and there was no improvement in the *TI* of l-HePC compared to that of f-HePC (Table IIB).

In vivo antitumor effect of doxorubicin encapsulating HePC liposomes. The *in vivo* effect of HePC-based liposomal doxorubicin on the growth suppression of xenografts was examined. Figure 1 shows the tumor growth rate of the tested formulations in each group. The growth of the tumors of the group treated with the doxorubicin solution was delayed compared to the control group and the minimum %ΔT/ΔC was 24.42 (experimental day 27). Both liposomal doxorubicin formulations (l-doxorubicin and l-HePC/doxorubicin) showed a faster tumor growth rate compared to the doxorubicin solution. The minimum %ΔT/ΔC was 27.0 and 24.36, respectively (experimental day 30) (Table III). The mice receiving either HePC solution or HePC liposomes showed little difference compared to the untreated mice. The tumor growth rate of liposomal or free HePC-treated animals was not significantly different when compared to the control (Figure 1).

Hexadecyl phoshocholine inhibits tumor growth, but its effect becomes very clear only after a 4-week treatment. As expected, free doxorubicin almost completely inhibits tumor growth after 2 weeks. The effect of the liposomal formulation ranked between the activity of free HePC and free doxorubicin. However of great interest was the notable cytotoxicity towards the tumor cells after a 4-week treatment The doxorubicin-containing formulations were found to be very active towards tumors, but also highly toxic for animals. Two out of 5 of the mice treated with doxorubicin solution (f-doxorubicin) died on experimental day 27 and the experiment was terminated on day 30, when all the mice of this group were found dead. One death (experimental day 23)

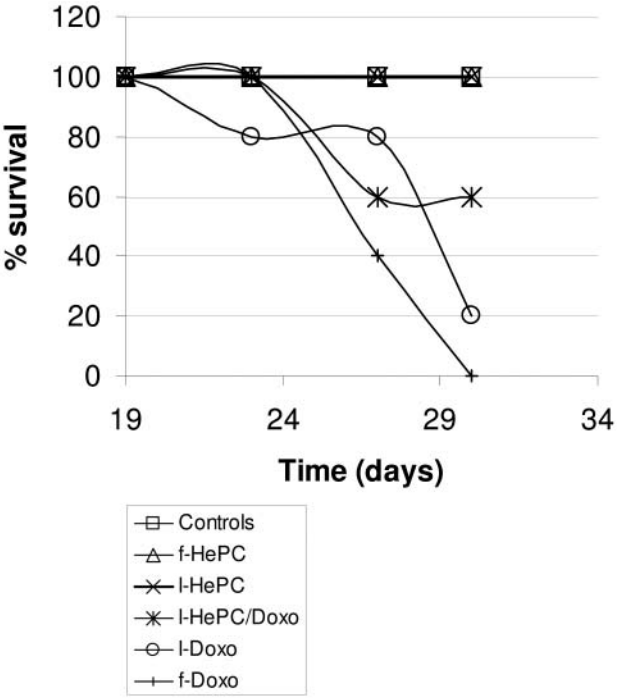

Figure 2. *Survival rates as a percentage (%) of the starting population for all formulations tested.*

was noticed in the group of mice treated with conventional liposomal doxorubicin (l-doxorubicin) and 3 more mice out of 5 were found dead on day 30. In the HePC-based liposomal doxorubicin-treated group (5 mice), 2 deaths were marked (experimental day 27). At the end of the experiment (day 30) the rest of the mice were alive (Figure 2). No significant changes in weight, appetite loss, or other side-effects were observed (Figure 3).

Discussion

Liposomal formulations of anthracyclines were linked to the reduction of their side-effects, improving the therapeutic index of the drug (6, 22, 23). HePC, which belongs to the class of ether lipid analogs of alkylphosphocholines, is in clinical use under the name miltefosine (9). Several studies describe liposomal preparations composed of alkylphosphocholines and HePC have been selected for preparing liposomes composed of cholesterol and dicetylphosphate (13). In this study, liposomes composed of HePC:EPC:SA were prepared using lipids of complementary molecular shapes, which can produce lamellars without adding cholesterol. This approach (24) is beneficial due to the reduction of the hemolytic activity of the ether lipids. Several studies have been published on the combination of ether lipids with other anticancer drugs or DNA-interactive agents (14, 15, 25). An attempt to combine

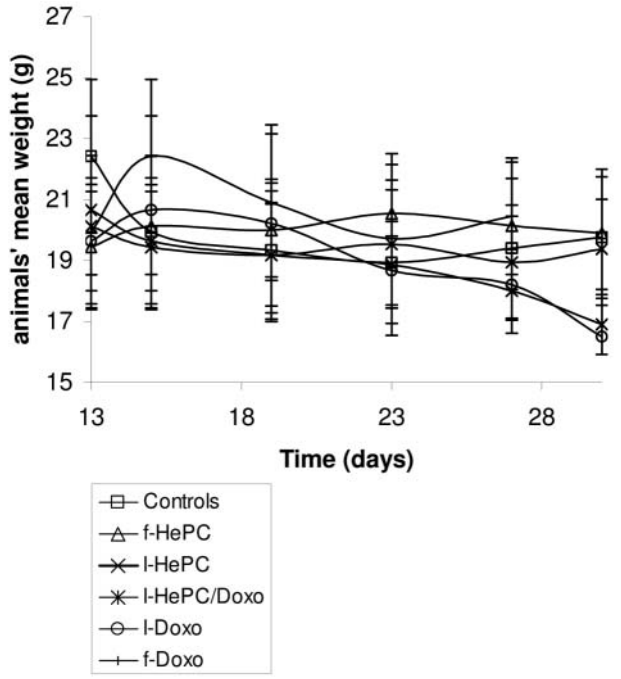

Figure 3. *Changes of the animal weight for the untreated (control) and treated mice. Measurements started on day 13 post-inoculation of cells and continued until the end of the experiment (day 30 post-inoculation) performed twice weekly.*

HePC with doxorubicin in a single liposomal formulation was performed in this study in order to evaluate the cytotoxic, as well as the antitumor, activity of the liposomal formulation against the HCT116 cell line and HCT116 xenograft *in vitro* and *in vivo*, respectively. As the results showed, the HePC:EPC:SA liposomes not encapsulating doxorubicin (liposomal formulation **1**) retained an *in vitro* cytotoxic activity almost identical to that of free HePC, probably due to the absence of cholesterol from the liposomal membrane that does not impede the interaction of HePC with the cell membrane (13). The apparent lack of *in vivo* activity of HePC in its free and liposomal forms can be attributed to the low dose used. This dose was much lower than doses used previously [(*e.g.*, 21 mg/kg (26) or 40.7 mg/ml (27)], but was chosen in order to be equal to that of the HePC content of the liposome **1** that encapsulated doxorubicin at a phospholipid to doxorubicin ratio of 0.96.

The *in vitro* activity of doxorubicin encapsulated into liposome **2** (l-doxorubicin) was reduced. On the contrary, liposomal formulation **1,** encapsulating doxorubicin (l-HePC/doxorubicin), showed better activity compared to that of liposomal formulation **2**. Despite the quick doxorubicin release, the therapeutic index of the liposome **1** (l-HePC/doxorubicin) was remarkably improved, as calculated from the activity against PBMCs *in vitro*.

The *in vivo* activity of free doxorubicin (f-doxorubicin), liposomal doxorubicin **1** and **2** (l-HePC/doxorubicin and l-doxorubicin) showed no statistical difference. However, fewer deaths were recorded for liposomal doxorubicin **1** than for liposomal doxorubicin **2** and for free doxorubicin, the latter being the most toxic preparation. No deaths were recorded for the free HePC (f-HePC) and liposomal HePC (l-HePC; liposomal formulation **1**, free of doxorubicin) treatments and of the control mice and, therefore, doxorubicin must have been responsible for the observed lethality. Previous studies using conventional liposomes encapsulating doxorubicin reported a comparable *in vivo* anticancer effect of free doxorubicin; the toxicity of l-doxorubicin (liposomal formulation **2,** encapsulating doxorubicin) was also comparable to that of f-doxorubicin, probably due to the high doxorubicin release (28). The release rate of the encapsulated doxorubicin is important and recently it has been suggested that the higher the release rate, the faster sufficient intercellular levels of doxorubicin will be achieved and the greater the cytotoxic effect will be (29). However, a slower release rate can be beneficial to reduce the toxicity of the doxorubicin liposomal formulation, since long circulating liposomes have an opportunity to accumulate in tumors and to exercise their cytotoxic effects (30).

The apparent reduction in toxicity of the l-HePC/ doxorubicin (liposomal formulation **1** encapsulating doxorubicin) in comparison to the l-doxorubicin (liposomal formulation **2**, encapsulating doxorubicin) can be attributed to the presence of HePC. This speculation is supported by the improvement of the uptake of the encapsulated doxorubicin by fatty-like lipid-containing liposomes (31).

To conclude, in this study, the cytostatic activity of rehydrated lyophilized hexadecyl phosphocholine (HePC) liposomes encapsulating doxorubicin was evaluated both *in vitro* and *in vivo* against a human colon cancer cell line and xenografts. The HePC- doxorubicin liposomes exhibited a pharmacological activity that was similar to that of the free doxorubicin, but their toxicity was lower than that of either free doxorubicin or HePC liposomes free of doxorubicin, as demonstrated on the mouse xenograft model. Therefore, the HePC/ doxorubicin liposomal formulation represents an interesting new drug delivery system for cancer treatment, mainly due to the apparent decreased toxicity.

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