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In-vitro cytotoxic/cytostatic activity of anionic liposomes containing vinblastine against leukaemic human cell lines

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Abstract

Liposomes prepared from lipids dipalmitoylphosphatidylcholine (DPPC) and dipalmitoylphosphatidylglycerol (DPPG) with cholesterol were used to investigate the percentage of vinblastine encapsulation and the influence of lipid composition on the retention properties of vinblastine in buffer as well as in cell culture medium. Differential scanning calorimetry (DSC) was applied, to study the effect of cholesterol on the phase transition temperature and on the ΔH of the two liposome formulations. The cytotoxic and cytostatic activity of the liposome-encapsulated vinblastine was also examined against six leukaemic human cell lines. The results showed that encapsulation of vinblastine into liposomes was greater than 98% with a drug–phospholipid molar ratio of 0.13–0.18. The major improvement in vinblastine retention in buffer as well as in culture medium was achieved by employing DPPG. The DSC data showed that vinblastine exerted a more perturbing effect in DPPC–cholesterol bilayers than in DPPG–cholesterol bilayers and this may explain their lower retention time. The 50% growth-inhibiting (GI50) and cytostatic (TGI) activity of liposomal vinblastine did not seem to be affected by the type of the liposome while the 50% cytotoxic activity (LC50) was affected in four out of the six cell lines tested. The parameters GI50, TGI and LC50 were estimated according to the instructions given by the NCI.

Introduction

Vinblastine is a mitotic inhibitor isolated from the plant *Catharanthus roseus* G. Don (Apocynaceae). It is used in the treatment of leukaemia, Hodgkin's disease, breast carcinoma, Wilm's tumor, Ewing's sarcoma and small-cell lung cancer, either alone or in combination with other chemotherapeutic agents (Carter & Livingston 1976). The mechanism of action of vinblastine is well established; it binds to spindle microtubules and arrests the metaphase of mitosis (Sieber et al 1976). Studies concerning the retention properties of vincristine in liposomal systems that have been evaluated in-vitro have resulted in improved retention in-vivo. The use of negatively charged lipids would be expected to increase the retention of the drug in liposomes (Maurer et al 1999). It is established that in-vivo the antitumour activity of liposomal vincristine correlates with the circulation lifetime of liposomes as well as with the capacity of the liposomes to retain entrapped drug (Mayer et al 1993). Differential scanning calorimetry (DSC) was applied as a means to explain the drug release results. This thermodynamic

technique is suitable to study the thermal effects of additives in membrane bilayers. The intermolecular interactions between the additives and the phospholipid bilayers determine the thermogram. Parameters utilized in a thermogram are the enthalpy of the transition (ΔH), the phase transition temperature (T_m) and the half-width of the phase transition ($T_{m\frac{1}{2}}$). The aim of this work was to study the retention of vinblastine in liposomes by using an anionic lipid (dipalmitoylphosphatidylglycerol; DPPG) in comparison with that of dipalmitoylphosphatidylcholine (DPPC), as well as to assess the cytotoxic and cytostatic activity of the two liposomal formulations against six human leukaemic cell lines in-vitro. The effect of the polar group of DPPG on the drug retention and the percentage of vinblastine encapsulation into liposomes was also investigated.

Materials and Methods

Materials

DPPC, DPPG and cholesterol were obtained from Avanti Polar Lipids Inc., AL. Vinblastine was used as its commercial form (vinblastine sulfate, Pharmachemie B.V., Haarlem, Netherlands). Chloroform and methanol were of spectroscopic grade. Ammonium sulfate, TES (*N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid) and RPMI-1640 culture medium were obtained from Sigma. Fetal calf serum was obtained from Biochrome, UK. MTT (3-(4,5-dimethylthiazol-2-yl)-2-5 diphenyl tetrazolium bromide) was purchased from Sigma-Aldrich.

Liposome preparation and vinblastine encapsulation

Two liposome formulations, DPPC–cholesterol (60:40) and DPPG–cholesterol (60:40), were prepared by the thin-film hydration method (Gabizon & Papahadjopoulos 1988). In brief, the lipid film was prepared by dissolving the lipid mixture in chloroform for DPPC–cholesterol and in chloroform–methanol (9:1) for DPPG–cholesterol. The solvent was subsequently slowly evaporated in a flash evaporator. Multilamellar vesicles (MLVs), were prepared by adding ammonium sulfate 150 mM (pH 5.3, 300 mOs) and then the liposomes were treated by freeze–thaw 5 times. The resultant vesicles were extruded ten times through two stacked polycarbonate membranes of 200-nm pore-size diameter using an extruder device (Lipex Biomembranes Inc., Canada) heated at 60°C (Olson et al 1979; Mayer et al 1985), to reduce the size of the vesicles and to obtain large unilamellar vesicles (LUVs). The final concentration of the formulations was 5 mg mL^{-1} in terms of total lipids. The size distribution histogram of liposomes was achieved by measuring the diameter of the resultant vesicles using a MASTERSIZER device (laser particle sizer) (Malvern Instruments Ltd). The vesicles were passed through Sephadex G-75 pre-equilibrated with 100 mM TES+100 mM NaCl (pH 7.5, 300 mOs), thus creating a transmembrane pH gradient. Subsequently, LUVs were incubated with 0.1 mL of vinblastine (5 mg mL^{-1}) at 28 and 60°C for various times. The vesicles with encapsulated vinblastine were separated from unentrapped vinblastine by filtration on a Sephadex G-75 column. The phosphorus was determined by phosphate assay (Morrison 1964) and the drug–lipid ratio was calculated.

Drug-release experiments

The release of vinblastine from liposomes in buffer was assayed. Vesicles containing vinblastine were first passed over a Sephadex G-75 column equilibrated with 100 mM TES+100 mM NaCl (pH 7.5, 300 mOs) to remove free drug and then placed in a plastic tube and incubated in a water bath maintained at 37°C. Samples were removed at various times and released vinblastine was separated employing a Sephadex G-75 column. Liposomes were disrupted with ethanol and the entrapped vinblastine was assayed by UV spectroscopy at 262 nm.

Determination of vinblastine released from liposomes in culture medium (RPMI-1640 medium supplemented with 10% heat-inactivated fetal calf serum and 2 mM L-glutamine) was performed whereby the liposomally entrapped vinblastine was diluted in 50% (v/v) culture medium and incubated in a water bath at 37°C. Samples were removed at various times and the released vinblastine was separated employing a Sephadex G-75 column. Retained vinblastine was analysed by HPLC after diluting the liposomes with isopropanol. All analyses were carried out by reverse-phase HPLC (RP-HPLC) using an isocratic system. In brief, a Nucleosil 100-S C18 (4.6 mm × 250 cm) stainless-steel column compiled with a GBC LC 1120 pump system with a GBC LC 1210 UV-vis detector was used, the chromatograms being monitored at 262 nm. The mobile phase was a mixture of methanol–acetonitrile 62% (4:1 v/v) and 38% buffer (14 mL diethylamine in 1 L of water, adjusted to pH 7.5 with *ortho*-phosphoric acid). The flow rate was 1.3 mL min^{-1} and the retention time was 10.5 min.

Differential scanning calorimetry (DSC)

Appropriate amounts of phospholipid with or without cholesterol at two concentrations (10 mol% and 30 mol%) were dissolved in spectroscopic-grade chloroform or chloroform–methanol. The solvent was evaporated by Rotavapore under vacuum (0.1 mmHg) at a temperature above the transition temperature. For measurements this dry residue was dispersed in appropriate amounts of bi-distilled water by vortexing. Portions of the samples (~ 5 mg) were sealed into stainless-steel capsules (Perkin Elmer). Thermograms were obtained on a Perkin Elmer DSC-7 calorimeter. Before scanning, the samples were held above their phase-transition temperature for 1–2 min to ensure equilibration. All samples were scanned at least twice until identical thermograms were obtained using a scanning rate of 2.5°C min^{-1}. The temperature scale of the calorimeter was calibrated using indium (T_m = 156.6°C) as standard sample.

Cytotoxic and cytostatic activity

The cytotoxic activity of liposomes (DPPC–cholesterol and DPPG–cholesterol) containing vinblastine were tested against the following cell lines: CCRF-CEM, MOLT4, HUT78 (T-cell line), RPMI 8226 (B-cell line), HL60 (promyelotic cell line) and K562 (proerythrocytes). All cell lines were grown as exponentially proliferating suspension cultures in RPMI-1640 medium supplemented with 10% heat-inactivated fetal calf serum, 2 mM L-glutamine and 50 μg mL^{-1} gentamycin, and incubated at 37°C in humidified atmosphere and 5% CO_2. The initial inoculation density ranged from 10 000 to 40 000 cells/well and was determined by taking into account cell mass and growth rate. Cell viability was assessed by trypan blue dye exclusion at the beginning of the experiment and was always greater than 98%. Cells were added at the appropriate inoculation density in 96-well microtitre plates and pre-incubated for 24 h in a moist atmosphere of 5% CO_2 in air at 37°C to allow stabilization before addition of the test formulations. To determine their activity, the liposome formulations (DPPC–cholesterol and DPPG–cholesterol (60:40)) were added at the same time to each cell line. Cultures containing buffer, 100 mM TES + 100 mM NaCl (pH 7.5, 300 mOs), were used as a negative control. After the addition of vinblastine encapsulated liposomes, as well as the empty liposomes, cells were cultured for 48 h in a moist 5% CO_2 atmosphere. Each formulation was inoculated at five concentrations (10^{-6} to 10^{-10} M). Empty liposomes were inoculated at a concentration corresponding to liposome-encapsulated vinblastine containing 10^{-6} M vinblastine. The activity of each formulation on each cell line was determined by the MTT assay. Briefly, 4 h before the end of the 48-h incubation period, MTT dissolved in PBS (phosphate-buffered saline) was added to the cell cultures to give a final concentration of 50 μg/well. At the end of the 48-h incubation period, dimethyl sulfoxide (DMSO) was added to the wells and the optical density was measured with an ANTHOS HT II Microelisa reader, using a test wavelength of 550 nm. The data represent the mean of three independent experiments run in triplicate and were analysed using a two-tailed Student's *t*-test. The following parameters were determined by our own customized software: GI50, TGI and LC50 (Monks et al 1991). Briefly, GI50 is the concentration where $100 \times (T - T_0)/(C - T_0) = 50$, and measures the growth inhibitory power of the tested compound. TGI is the concentration of the test compound where $100 \times (T - T_0)/(C - T_0) = 0$ and measures the cytostatic effect. Finally, LC50 is the concentration of the drug where $100 \times (T - T_0)/T_0 = -50$ and measures the cytotoxic effect of the compound. T is the optical density of the test well after the 48-h period of exposure to test compound, T_0 is the optical density at time zero (when the compound was added) and C is the optical density of the control well (cell incubated for 48 h with no additives) (Maswadeh et al 2000). Liposomes were also tested against peripheral blood mononuclear cells (PBMCs) isolated from normal human donors.

Results and Discussion

Vesicle size distribution

The vesicle size distribution for the liposomes (DPPC–cholesterol and DPPG–cholesterol) was determined by laser particle sizer (MASTERSIZER). Size distribution histogram indicated that repetitive extrusion of MLVs systems through two stacked polycarbonate filters with 200-nm pore size after five freeze–thaw cycles resulted in LUV systems exhibiting a relatively homogeneous size distribution. The volume distribution mean of the two liposomal formulations were 240 $\pm$ 147 nm for DPPC–cholesterol and 240 $\pm$ 155 nm for DPPG–cholesterol, according to Malvern software (MASTERSIZER software), with 50% of distribution percentiles at 210 nm for both formulations, using the polydisperse analysis and 3NAD presentation.

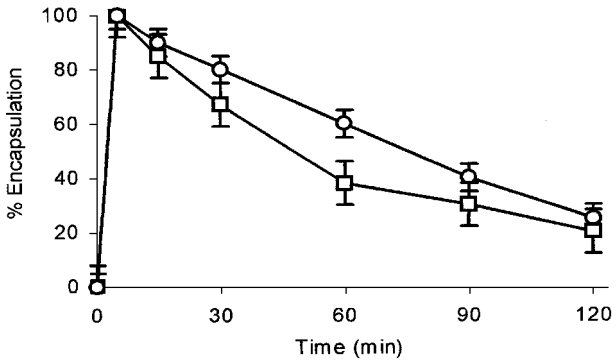

Figure 1 Uptake of vinblastine into DPPC–cholesterol (□) or DPPG–cholesterol (○) liposomes at 60°C. Each point represents the mean of three independent experiments (bars represent s.d.).

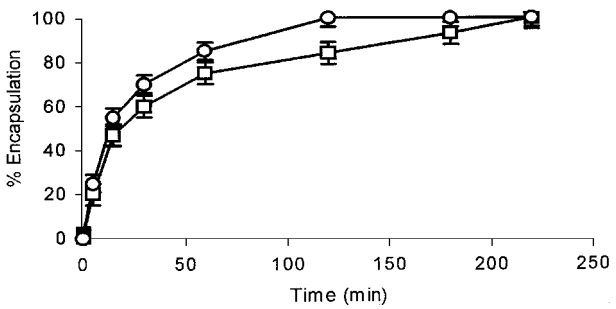

Figure 2 Uptake of vinblastine into DPPC–cholesterol (□) or DPPG–cholesterol (○) liposomes at 28°C. Each point represents the mean of three independent experiments (bars represent s.d.).

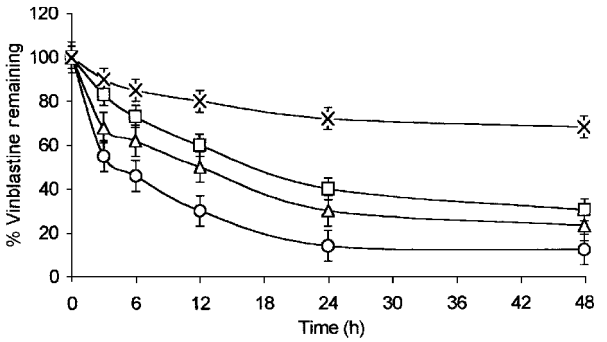

Figure 3 Release of vinblastine from DPPC–cholesterol (□) or DPPG–cholesterol (X) liposomes at 37°C in buffer (100 mM TES+ 100 mM NaCl; pH 7.5, 300 mOs) and release of vinblastine from DPPC–cholesterol (○) and DPPG–cholesterol (△) liposomes at 37°C in culture medium (RPMI-1640 medium supplemented with 10% heat-inactivated fetal calf serum and 2 mM L-glutamine) (50% v/v). Each point represents the mean of three independent experiments (bars represent s.d.).

Encapsulation and release of vinblastine in liposomes containing cholesterol

Vinblastine was encapsulated into liposomes (DPPC–cholesterol and DPPG–cholesterol (60:40)) in response to a transmembrane ammonium sulfate gradient with trapping efficiencies more than 98% in all cases with a drug–phospholipid molar ratio as follows: 0.13 for DPPG–cholesterol incubated at 28°C for 120 min; 0.18 for DPPG–cholesterol incubated at 60°C for 5 min; 0.14 for DPPC–cholesterol incubated at 28°C for 225 min; and 0.17 for DPPC–cholesterol incubated at 60°C for 5 min. The rate and extent of vinblastine uptake was highly dependent on temperature and lipid composition. Figure 1 shows that the rate of vinblastine uptake was fast for the two liposomal formulations at 60°C, considering that more than 98% of vinblastine was encapsulated into the liposomes within 5 min. Also Figure 1 shows that vinblastine was released from DPPC–cholesterol and DPPG–cholesterol vesicles following uptake at high incubation temperature (60°C). More specifically, at 60°C, more than 30% and 20% of the entrapped vinblastine was released from DPPC–cholesterol and DPPG–cholesterol, respectively, within 30 min. To decrease leakage from liposomes during the process of vinblastine encapsulation, the incubation temperature was decreased to 28°C. Figure 2 shows that more than 98% vinblastine encapsulation into DPPG–cholesterol and DPPC–cholesterol liposomes could be achieved within 120 min and 225 min, respectively, at 28°C.

Retention of vinblastine within liposomes

The retention of vinblastine within liposomes was monitored at 37°C in buffer (100 mM TES+100 mM NaCl; pH 7.5, 300 mOs) and in 50% v/v culture medium (RPMI-1640 medium supplemented with 10% heat-inactivated fetal calf serum and 2 mM L-glutamine) (Figure 3). The release of vinblastine, within 48 h, from DPPC–cholesterol in buffer was 70% and from DPPG–cholesterol was 25%. When liposomal formulations were exposed to culture medium 50% (v/v), more than 45% of vinblastine was released from DPPC–cholesterol and from DPPG–cholesterol within 6 h and 12 h, respectively (Figure 3).

Thermal effects of vinblastine in phospholipid bilayers containing cholesterol

Hydrated DPPC and DPPG phospholipids spontaneously formed bilayers. The bilayers existed in the gel phase at temperatures lower than 35°C and in the

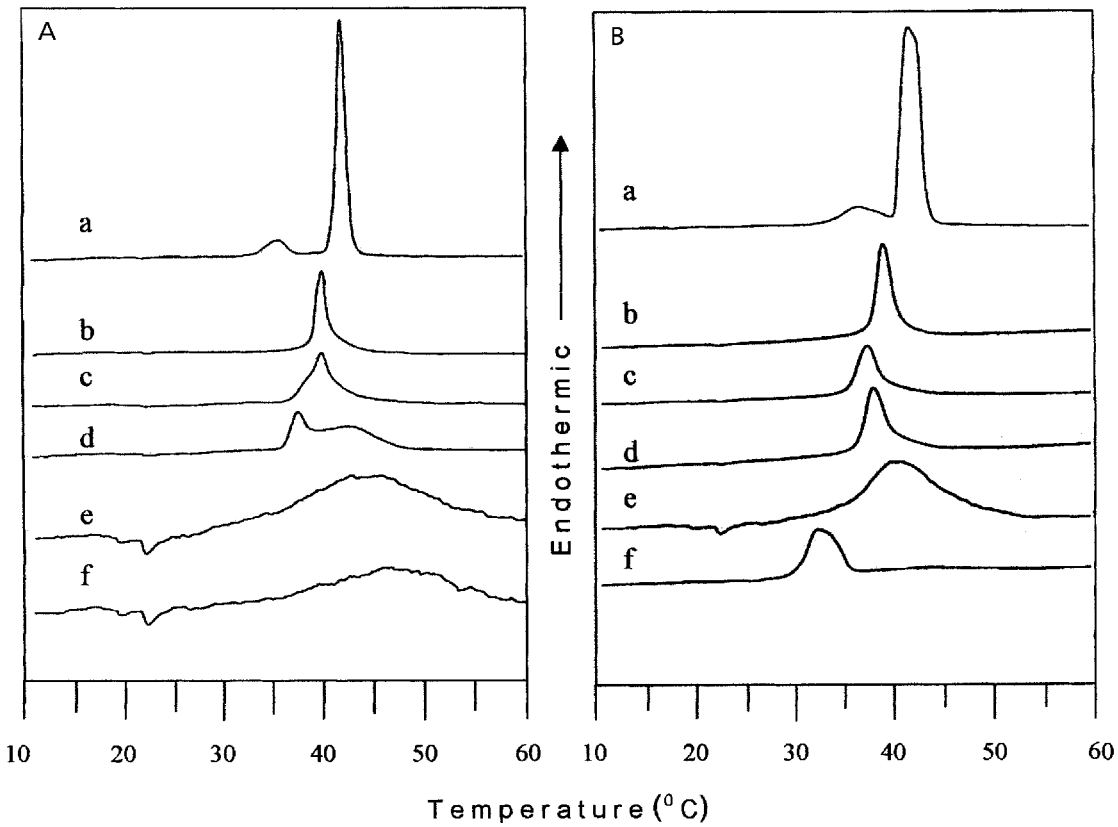

Figure 4 A. DSC calorimetry scan of: DPPC (a); DPPC–cholesterol (x = 0.10) (b); DPPC+cholesterol (x = 0.10)+vinblastine (x = 0.045) (c); DPPC+cholesterol (x = 0.10)+vinblastine (x = 0.17) (d); DPPC–cholesterol (x = 0.30) (e); and DPPC–cholesterol (x = 0.30) +vinblastine (x = 0.17) (f). B. DSC calorimetry scan of: DPPG (a); DPPG–cholesterol (x = 0.10) (b); DPPG+cholesterol (x = 0.10)+vinblastine (x = 0.045) (c); DPPG+cholesterol (x = 0.10)+vinblastine (x = 0.17) (d); DPPG–cholesterol (x = 0.30) (e); and DPPG–cholesterol (x = 0.30)+vinblastine (x = 0.17) (f). x represents molar ratio.

liquid crystalline phase at temperatures higher than 42°C (Figure 4). The transition was accompanied by several structural changes in the lipid molecules as well as by systemic alterations in the bilayer geometry, but the most prominent feature was the trans-gauche isomerization taking place in the acyl chain conformation. The addition of x = 0.10 (molar ratio) cholesterol in DPPC and DPPG phospholipid bilayers results in abolishment of the pre-transition, lowering of ΔH and marginal decrease in T_m (Estep et al 1978; Mavromoustakos & Daliani 1999). The presence of x = 0.45 vinblastine has more drastic effects in DPPC–cholesterol bilayers as it causes inhomogeneity in the membrane and increase in the breadth of phase transition. This effect is more pronounced when a higher cholesterol concentration is used (x = 0.17). At higher cholesterol content the presence of vinblastine in DPPC–cholesterol bilayers caused decrease of T_m and breadth of phase transition while in DPPG–cholesterol bilayers almost no effect was discernible (Table 1). These thermal effects of vinblastine in the two bilayers may explain their different retention properties in-vitro and in-vivo.

Cytotoxicity and cytostaticity of vinblastine

The two liposome formulations containing vinblastine were tested for their cytotoxic activity against six leukaemic cell lines at five 10-fold dilutions with an initial concentration of 2.5×10^{-5} M of vinblastine. The two liposomes were also tested against resting and phytohaemagglutinin-P-activated peripheral blood mononuclear cells (PBMCs) isolated from normal human donor. Vinblastine was found to be inactive at all concentrations tested, independently of the kind of liposome. For each leukaemic cell line, GI50 (growth-inhibiting activity), TGI (cytostatic activity) and LC50 (cytotoxic activity) were estimated and the results,

Table 1 Values of pretransition temperature ($T_{pretrans}$), half-width temperature ($T_{m\frac{1}{2}}$), peak temperature ($T_{m\frac{1}{2}}$), peak temperature (T_m) and enthalpy change (ΔH) of phospholipid bilayers without and with vinblastine and phospholipid–cholesterol without or with vinblastine.

Samples	$T_{pretrans}$	T_m (°C)	$T_{m\frac{1}{2}}$ (°C)	ΔH (cal g^{-1})
DPPC	34.8	41.2	2.8	1.11±0.04
			1.0	9.96±0.08
DPPC+cholesterol (x = 0.10)		39.5	1.1	6.65±0.44
DPPC+cholesterol+vinblastine (x = 0.045)		38.9	3.0	8.11±0.56
DPPC+cholesterol+vinblastine (x = 0.17)		36.2	7.5	8.31±0.32
DPPC+cholesterol (x = 0.3)		41.7	16	3.02±0.15
DPPC+cholesterol+vinblastine (x = 0.17)		44.9	17.5	1.69±0.26
DPPG	36.3	40.7	5.0	1.08±0.02
			2.25	10.74±0.04
DPPG+cholesterol (x = 0.10)		38.0	1.9	7.97±0.50
DPPG+cholesterol+vinblastine (x = 0.045)		35.9	2.5	5.54±0.24
DPPG+cholesterol+vinblastine (x = 0.17)		36.7	2.5	7.48±0.27
DPPG+cholesterol (x = 0.30)		39.2	8.5	4.53±0.13
DPPG+cholesterol+vinblastine (x = 0.17)		31.8	4.0	4.88±0.04

Table 2 GI50, TGI and LC50 of vinblastine encapsulated in liposomes.

Cell lines	Vinblastine in DPPC–cholesterol liposomes			Vinblastine in DPPG–cholesterol liposomes		
	GI50	TGI	LC50	GI50	TGI	LC50
CCRF-CEM	2.5[a]	4.3±0.6	7.3±0.7	2.5	10.7±1.3	25.5±3.3
MOLT4	3.0±0.3	6.6±0.6	10.1±0.8	2.5	8.7±0.9	34.6±3.2
HUT78	2.5	2.5	7.8±0.3	15.4±2.3	29.0±3.0	42.6±5.1
RPMI8226	2.5	2.5	6.4±0.6	2.5	2.5	57.4±6.0
K562	3.0±0.4	7.5±0.6	100.0±11.0	2.5	25.9±3.9	50.4±5.1
HL60	12.2±1.8	18.8±1.9	25.5±2.0	6.9±0.9	14.5±1.5	22.1±3.3

GI50 is the concentration where $100\times(T - T_0)/(C - T_0) = 50$, and measures the growth inhibitory power of the tested compound. TGI is the concentration of the test compound where $100\times(T - T_0)/(C - T_0) = 0$ and measures the cytostatic effect. LC50 is the concentration of the drug where $100\times(T - T_0)/T_0 = -50$ and measures the cytotoxic effect of the compound. T is the optical density of the test well after the 48-h period of exposure to test compound, T_0 is the optical density at time zero (when the compound was added) and C is the optical density of the control well (cell incubated for 48 h with no additives) (Maswadeh et al 2000). [a]Not tested at lower doses. Results are expressed in nM. Values represent means of three independent experiments done in triplicate±s.d. Data were evaluated using a two-tailed unpaired (for each liposomal formulation) and paired (for each cell line) *t*-test ($P \leqslant 0.05$).

expressed in nM, are summarized in Table 2. The mean graphs for TGI are also presented in Figure 5.

With the exception of HUT 78 cells, which was more resistant to vinblastine encapsulated in DPPG–cholesterol liposomes in all three parameters calculated, the growth-inhibiting (GI50) activity of vinblastine was not affected by the type of the liposome within which the vinblastine was encapsulated. On the other hand, vinblastine exhibited a lesser cytostatic and cytotoxic activity (as expressed by TGI and LC50 calculated parameters, respectively) when encapsulated in DPPG–cholesterol liposomes. These differences between the two liposomal formulations were not of major significance, however, as they were largely flattened when mean graphs for TGI (Figure 5) and LC50 (not shown) were plotted, with the aforementioned exception of HUT 78 cells.

The reduced cytostatic and cytotoxic activity of vinblastine encapsulated within DPPG–cholesterol liposomes could be explained by taking into account the improved retention properties of these liposomes compared with those of DPPC–cholesterol. The greater

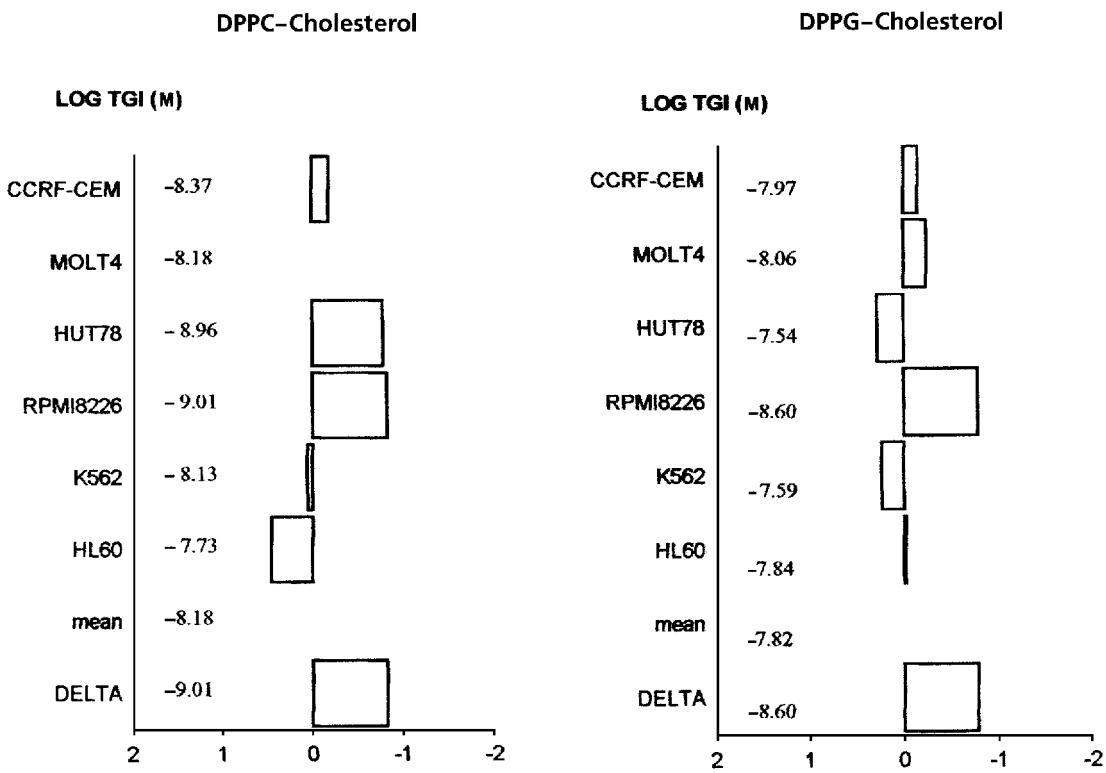

Figure 5 The mean graph of log TGI (cytostatic effect) for vinblastine encapsulated within DPPC–cholesterol or DPPG–cholesterol liposomes tested against six human cell lines. TGI is the concentration of test compound where $100\times(T - T_0)/(C - T_0) = 0$; T is optical density of test well after 48 h exposure to test compound, T_0 is optical density at time zero and C is optical density of control.

release of vinblastine from the latter (more than 45% within the first 6 h) contributes to the longer exposure of the cells to a higher concentration of free vinblastine. However, the prolonged exposure time of cells to free vinblastine could result in a lower concentration being required to achieve the same cytotoxic effect, a phenomenon observed especially in drugs acting during mitosis, as does vincristine (Boman et al 1995).

Conclusion

In this study, vinblastine was encapsulated into liposomes using the ammonium sulfate gradient-loading method, with an efficiency of more than 98%. A major improvement in vinblastine retention in buffer, as well as in culture medium, was achieved by employing DPPG. When liposomes were incubated in culture medium (50% v/v) instead of buffer, increased leakage was displayed at 37°C producing release of more than 45% of vinblastine from DPPC–cholesterol liposomes within the first 6 h. It is worthy of note that DPPC and DPPG have different polar groups with the same saturated acyl chain length. Previous studies using doxorubicin (Cullis et al 1997) and vincristine (Boman et al 1995, 1998) have shown that drug retention after loading in response to a ΔpH (inside acidic) is enhanced in DSPC–cholesterol LUVs in comparison with egg phosphatidylcholine–cholesterol LUVs.

Since the acyl chain length of DPPC and DPPG are identical and the phase transition temperature varies by 0.5°C it is proposed that the improvement in the retention properties of DPPG is due to the negatively charged polar group of the DPPG.

Vinblastine, which is positively charged, could be electrostatically bound to the negatively charged polar group of the DPPG to produce an increase of vinblastine membrane–water partitioning (Maurer et al 1999). In many liposomal formulations which contain doxorubicin, daunorubicin or epirubicin bound to negatively charged head-group of phospholipids such as phosphatidylserine, phosphatidylglycerol or cardiolipin, the COO^- group of the phospholipid is known to form a very stable complex with the NH_3^+ group of anthracycline at lower values of pH (Lasic 1993).

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