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Identification of vesicle properties that enhance the antitumour activity of liposomal vincristine against murine L1210 leukemia

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Abstract. The influence of vesicle lipid composition, size and drug-to-lipid ratio on the antitumour activity of liposomal vincristine was assessed in the murine L1210 ascitic leukemia model. A pH gradient-dependent entrapment procedure was used to encapsulate vincristine and allowed such vesicle properties to be independently varied. Free vincristine delivered i.v. at the maximum tolerated dose (2.0 mg/kg) resulted in a 27.8% increase in the life span (ILS) of mice inoculated i.p. with L1210 cells. Encapsulation of the drug in egg phosphatidylcholine/cholesterol vesicles did not significantly increase the antitumour efficacy of vincristine (ILS, 38.9%). In contrast, administration of vincristine entrapped in vesicles composed of distearoylphosphatidylcholine (DSPC)/cholesterol resulted in ILS values as high as 133%. This enhanced antitumour activity of the DSPC/cholesterol formulations was sensitive to the size of the liposomes; increasing the vesicle size from 100 nm to 1 μm decreased the ILS from 133.3% to 55.6% at a drug dose of 2.0 mg/kg. Decreasing the drug-to-lipid ratio from 0.1:1 to 0.05:1 (w/w) had negligible effects on the activity of liposomal vincristine; however, a further decrease in the drug-to-lipid ratio to 0.01:1 (w/w) decreased the antitumour potency at all drug doses studied. Pharmacology studies indicated that the antitumour activities of free and various liposomal forms of vincristine correlated well with the residence time of the drug in the circulation. These studies indicate that efforts to enhance the therapeutic activity of vincristine through liposome encapsulation must address not only the circulation lifetime of the vesicle systems but also the capacity of the liposomes to retain entrapped drug in vivo.

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Abbreviations: HPLC, high-pressure liquid chromatography; MLV, multilamellar vesicle; EPC, egg phosphatidylcholine; DSPC, distearoylphosphatidylcholine; CHOL, cholesterol; VINC, vincristine; HBSS, Hanks' balanced salt solution

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Introduction

The use of liposomes to improve the therapeutic index of agents employed in cancer chemotherapy has gained increasing interest [12, 28]. This approach is best exemplified in the case of doxorubicin. Several studies have shown that liposome-mediated alterations in the pharmacokinetic and biodistribution properties of this drug yield decreased toxicities such as cardiotoxicity and nephrotoxicity [7, 8, 10, 13, 23, 27, 30, 32]. As a result, higher drug doses can be applied, leading to an increase in antitumour efficacy. In this context, we have previously described the use of transmembrane pH gradients to encapsulate efficiently high levels of vincristine inside liposomes with well-defined physical characteristics for therapeutic applications [20]. This technique allows trapping efficiencies approaching 100%, stable drug retention and high drug-to-lipid ratios. Furthermore, initial evaluations in animal models indicate that these systems are less toxic and more efficacious than free vincristine [26].

Previous studies have demonstrated that vesicle size, lipid composition and drug-to-lipid ratio significantly influence the toxicity and antitumour efficacy behaviour of liposomal doxorubicin [8, 23]. Such effects appeared related to liposome-dependent changes in the drug's pharmacological behaviour. In the present studies, we investigated the influence of vesicle size, lipid composition and drug-to-lipid ratio on the antitumour efficacy of liposomal vincristine. Blood clearance evaluations were also performed in an effort to relate the antitumour activity of these formulations to the pharmacological behaviour of the drug.

Materials and methods

Oncovin (vincristine sulfate) was obtained from the British Columbia Cancer Agency (Vancouver, B.C.). Tritiated cholesteryl hexadecylether was purchased from New England Nuclear and was >95% pure. Tritiated vincristine was purchased from Amersham (Oakville, Ontario). Purity assessment and bulk purification (when necessary) of radiolabeled vincristine was completed by HPLC within 24 h prior to its use. This was achieved employing a 150- × 4.9-mm C-18 column (World Wide Moni-

toring Corp., Horsham, Pa.) with a methanol : 10 m*M* ammonium sulfate gradient (50 : 50 – 90 : 10, v/v). EPC and DSPC were purchased from Avanti Polar Lipids and were >99% pure. Cholesterol and all salts were obtained from Sigma Chemical Company (St. Louis, Mo.). Female DBA/2J mice (6 – 8 weeks old) were purchased from Jackson Laboratory Animals.

EPC/cholesterol (molar ratio, 55 : 45) or DSPC/cholesterol (molar ratio, 55 : 45) lipid films were prepared by vacuum evaporation from a $CHCl_3$ solution. Lipids were then hydrated in 300 m*M* citric acid (pH 4.0) by vortex mixing using a lipid : buffer ratio of 100 mg/ml. The MLVs were frozen and thawed five times [22] and then extruded ten times through polycarbonate filters of the indicated pore size [21] employing a lipid extrusion device obtained from Lipex Biomembranes (Vancouver, B.C.). Production of the DSPC/cholesterol samples used a Thermobarrel Extruder equilibrated at 60° C. Mean vesicle diameters were determined by quasielastic light scattering (employing a Nicomp 370 particle sizer). Vincristine was entrapped in the liposomes by adding vesicles (100 mg/ml) to the Oncovin solution (1 mg vincristine/ml) to achieve the indicated drug-to-lipid ratios. The pH of the sample was then raised to 7.0 – 7.2 with 0.5 *M* Na_2HPO_4, and the sample was subsequently heated at 60° C for 10 min unless otherwise indicated. Vincristine entrapment was determined by column chromatography techniques [26] employing 297-nm uvspectroscopy ($Abs._{297}$; in ethanol : H_2O; 8 : 2, v/v) and $Abs._{815}$ spectroscopic assays for quantitation of vincristine and lipid, respectively. Initial drug-to-lipid ratios were determined prior to the alkalinization step. Analysis by HPLC employing radiolabeled and non-radiolabeled drug indicated that decomposition of vincristine during encapsulation or upon storage prior to its in vivo use was negligible (>95% purity).

The antitumour activity of free and liposomal vincristine was assessed employing the L1210 lymphocytic leukemia models. DBA/2J mice (6 – 10 mice/group) were injected i.p. with 1×10^5 L1210 cells (obtained from the NCI tumour bank) derived from the ascites fluid of a previously infected mouse. The indicated doses of saline, free vincristine and liposomal vincristine were given i.v. at 24 h after tumour inoculation, and animal weights as well as mortality rates were monitored. Increase in life span (ILS) values were calculated from the median survival times. Mean and median survival times and the statistical significance of the results were determined employing a two-tailed Wilcoxon's ranking test (randomized two-group design). It should be noted that results similar to those reported herein for DBA/2J mice are obtained employing the L1210 model grown in BDF_1 mice. Similar trends in %ILS are observed; however, the maximal therapeutic dose is elevated and no long-term survival is observed for this mouse strain (data not shown).

For cytotoxicity assays, purified L1210 cells were centrifuged at 1200 rpm for 5 min and counted. Dilutions were made in RPMI 1640 to obtain concentrations such that the final cell concentration was 10^6 cells/ml for variable time-exposure experiments and 5×10^4 cells/ml for 3-day continuous-exposure experiments. Stock solutions of drugs were serially diluted in RPMI 1640 to give final concentrations of vincristine in the cell culture ranging from 10^{-4} to 10^2 μg/ml. In the 3-day continuous-exposure experiments, 0.5 ml of cells (1×10^5 cells/ml) was dispersed into each well (24-well plates), and 0.5 ml of varying drug concentrations was added. In the variable time-exposure experiments, the cells (2×10^6/ml) were mixed (1 : 1, v/v) with varying drug concentrations in 6-well plates, exposed to the drug for selected intervals (1, 4, 6 and 24 h at 37° C, washed twice by centrifugation in HBSS at 1200 rpm for 5 min and diluted in RPMI to yield 5×10^4 cells/ml per well. The cells were subsequently placed in an incubator for 3 days and the number of viable cells were determined in each well. Each set of triplicate experiments was repeated at least two times. All experiments included control cells, which were handled identically as the treated cells and plated into triplicate wells.

Plasma clearance studies were performed by injecting four mice (18 – 22 g) per time point with the indicated doses of free or liposomal vincristine via a lateral tail vein. At the indicated times, blood was collected from anaesthetized mice via heart puncture and placed into Microtainer tubes containing ethylenediaminetetraacetic acid (EDTA) beads (Becton Dickenson). Plasma samples were obtained by pelleting the blood cells via centrifugation (500 *g* for 10 min). Samples were then assayed for radioactivity by scintillation counting (counting efficiencies in excess of 25%). For liposomal vincristine preparations, identical samples were prepared containing either radiolabeled vincristine or lipid to allow both the drug and the lipid components to be monitored in complementary experiments.

Results

Characterization of liposomal vincristine preparations

Liposomal vincristine preparations used for in vivo assessment were characterized with respect to size, trapping efficiency and drug-to-lipid ratio. In addition, drug release from the vesicles and alterations in the transmembrane pH gradient were monitored in the presence of blood. By employing the extrusion and pH gradient drug-loading techniques for liposome production and vincristine entrapment, respectively, liposomal vincristine preparations differing in only one physical characteristic can be generated. Liposomal vincristine systems can be readily prepared for EPC/cholesterol and DSPC/cholesterol for liposome sizes ranging from 0.1 to 2 μm and drug-to-lipid ratios ranging from 0.01 : 1 to 0.1 : 1. The drug-trapping efficiencies of all liposomal vincristine preparations investigated in the present studies are in excess of 95%. It should be noted that the drug-to-lipid ratios employed in our study are lower than the 0.2 : 1 (w/w) ratio previously used for pH gradient-generated liposomal vincristine systems, as the 120-nm vesicles evaluated in the present studies have a reduced buffering capacity as compared with the 230-nm vesicles used in the prior study [26]. At a drug-to-lipid ratio of 0.2 : 1 (w/w) the uptake process depletes a significant portion of the entrapped proton pool in the 120-nm vesicle systems and the trapping efficiencies decrease (data not shown; see [24] for a detailed description).

We have previously demonstrated that release of entrapped vincristine is sensitive to the lipid composition of the vesicle [26]. Since drug release may be expected to be a major factor in determining the biological activity of liposomal vincristine, the kinetics of vincristine leakage from the vesicles and depletion of the transmembrane pH gradient were studied in vitro in the presence of blood at 37° C. As shown in Fig. 1 A, EPC/cholesterol liposomal vincristine preparations rapidly release vincristine upon exposure to blood, resulting in 76% and 96% drug leakage after 1- and 24-h incubations, respectively. This loss of entrapped drug is accompanied by a concomitant reduction in the magnitude of the pH gradient (Fig. 1 B). In contrast, DSPC/cholesterol preparations release 20% or less of the encapsulated vincristine over 1 h and exhibit an increase in the stability of the pH gradient. Decreasing the drug-to-lipid ratio of these systems decreases the rate of drug release from the liposomes.

Antitumour activity of free and liposomal vincristine preparations

Antitumour evaluations of free vincristine and liposomal vincristine preparations were based on the extension of

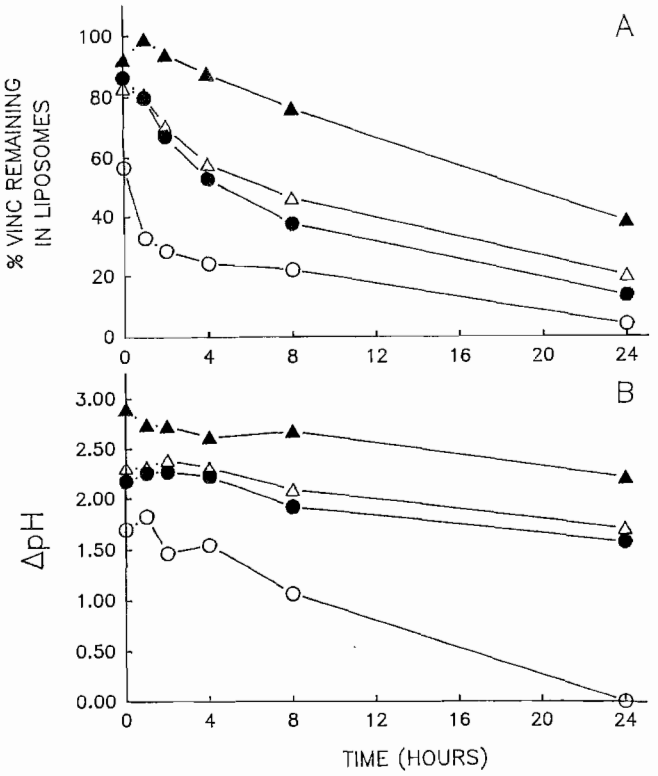

Fig. 1. A Vincristine retention and **B** transmembrane pH gradient in blood at 37° C as determined for 120-nm EPC/cholesterol (○) and 120-nm DSPC/cholesterol (●) liposomal vincristine preparations displaying a drug-to-lipid ratio of 0.1 : 1 (w/w). DSPC/cholesterol systems displayed drug-to-lipid ratios of 0.1 : 1 (●), 0.05 : 1 (△) and 0.01 : 1 (▲). Dilutions into blood were performed to achieve a final drug concentration of 0.04 mg/ml

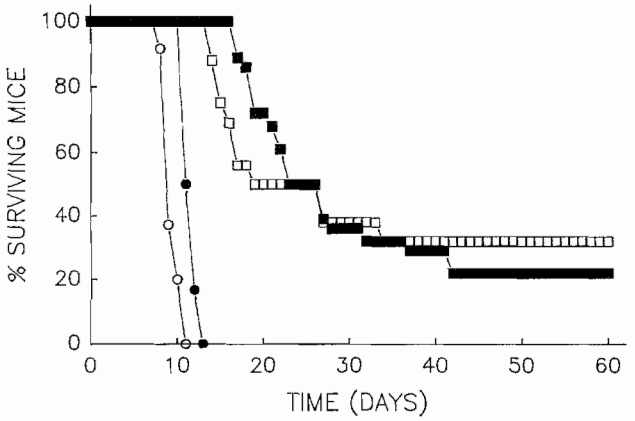

Fig. 2. Survival curves generated for DBA/2J mice inoculated i.p. with L1210 cells and treated 24 h later by i.v. injection of saline (n = 24, ○), free vincristine at a dose of 2 mg/kg (n = 12, ●) and 120-nm DSPC/cholesterol-entrapped vincristine at doses of 2 mg/kg (n = 28, ■) and 3 mg/kg (n = 16, □). Liposomal vincristine systems were prepared at a drug-to-lipid ratio of 0.1 : 1 (w/w)

survival time provided by i.v. administration of the treatments at 24 h after i.p. inoculation of L1210 cells into DBA/2J mice. In the absence of any treatment, the mice typically die between day 8 and day 11 (see Fig. 2). Administration of empty liposomes does not alter the time frame of death in this tumour model (data not shown). Free vincristine injected at a dose of 1 mg/kg provides no significant therapeutic effect as indicated by an ILS value of 5.5% (Table 1). Increasing the dose of free vincristine to 2 mg/kg (maximal therapeutic dose) results in a small but statistically significant (P <0.01) increase in antitumour activity with an ILS value of 27.8% (Fig. 2, Table 1). It should be noted that in previous studies [26] as well as unpublished observations by the present authors, attempts to improve the antitumour activity of free vincristine through the use of a wide variety of multiple dose regimens have been unsuccessful.

Previous investigations with doxorubicin [23] have shown that of the three major liposome physical charac-

Table 1. Effect of vesicle size on the antitumour activity of liposomal vincristine

Group	Dose (mg/kg)		Survival time (days)			ILS (%)	L/F[b]
	Drug	Lipid	Mean	Median	60-Day		
Control	–	–	9.6	9.0	0/24	–	–
Free VINC	1	–	9.6	9.5	0/6	5.5*	–
	2	–	11.7	11.5	0/12	27.8*	–
1 μm (0.5–2.0 μm)	1	10	12.3	11.5	0/6	27.8*	1.21
DSPC/CHOL-VINC	2	20	14.5	14.0	0/6	55.6*	1.22
0.6 μm (0.3–1.0 μm)	1	10	13.2	13.5	0/6	50.0*	1.42
DSPC/CHOL-VINC	2	20	14.8	15.0	0/6	66.7*	1.30
0.2 μm (0.220 μm)	1	10	14.5	14.5	0/6	61.1	1.52
DSPC/CHOL-VINC	2	20	N/A[a]	20.0	1/6	122.2	1.74
0.1 μm (0.120 μm)	1	10	15.7	16.0	0/12	77.8	1.68
DSPC/CHOL-VINC	2	20	N/A[a]	21.0	1/12	133.3	1.83

[a] Mean survival times were not calculated for groups exhibiting 60-day survivors due to the artificial designation of the day of death for these animals

[b] L/F values represent the median survival time for the liposomal treatment group divided by the median survival time for the group given the free drug at an equivalent dose

* Significantly different (P <0.05) from results obtained using 0.1-μm-sized DSPC/cholesterol-entrapped vincristine at the equivalent drug dose

Table 2. Effect of lipid composition of the antitumour activity of liposomal vincristine

Group	Dose (mg/kg)		Survival time (days)			ILS (%)[a]	L/F[b]
	Drug	Lipid	Mean	Median	60-Day		
EPC/CHOL-VINC[c]	1	10	11.3	11.0	0/6	22.2	1.16
	2	20	12.2	12.5	0/6	38.9	1.09
DSPC/CHOL-VINC[c]	1	10	15.7	16.0	0/12	77.8	1.68*
	2	20	N/A[d]	21.0	1/12	133.3	1.83*

[a] Values for % ILS were calculated on the basis of the median survival times as indicated in Materials and methods
[b] L/F values reflect the median survival time for the liposomal vincristine group divided by the median survival time for the group given the free drug at the same dose
[c] Vincristine was encapsulated inside liposomes extruded through 100-nm pore-size filters at a drug-to-lipid ratio of 0.1 : 1 (w/w)
[d] Mean survival times were not determined due to long-term survivors
* Significant at the $P < 0.05$ level

Table 3. Effect of drug-to-lipid ratio on the antitumour activity of liposomal vincristine

Group	Dose (mg/kg)		Survival time (days)			ILS (%)
	Drug	Lipid	Mean	Median	60-Day	
Control	–	–	9.6	9.0	0/24	–
120 nm DSPC/CHOL-VINC[a]	1	10	15.7	16.0	0/12	77.8
	2	20	N/A[d]	21.0	1/12	133.3
120 nm DSPC/CHOL-VINC[b]	1	20	15.8	16.0	0/22	77.8
	2	40	N/A[d]	23.0	6/28	155.6*
120 nm DSPC/CHOL-VINC[c]	1	100	15.8	15.0	0/12	66.7
	2	200	18.8	18.0	0/12	100.0*

Vincristine was encapsulated in 126-nm DSPC/cholesterol vesicles at a drug-to-lipid weight ratio of [a]0.1 : 1, [b]0.05 : 1 and [c]0.01 : 1
[d] Mean survival times were not determined due to long-term survivors
* Significantly different from values obtained using an equivalent dose of vincristine delivered in DSPC/cholesterol systems at a drug-to-lipid ratio of 0.1 : 1 (*$P < 0.05$, **$P < 0.01$)

teristics (size, lipid composition and drug-to-lipid ratio), only vesicle size dramatically alters the antitumour efficacy of liposomal doxorubicin. Specifically, decreasing the vesicle size results in an increase in the therapeutic activity of this agent. The role of vesicle size in determining the efficacy of liposomal vincristine preparations composed of DSPC/cholesterol was therefore studied to identify the possibility of similar relationships. As shown in Table 1, decreasing the vesicle size from approximately 1 μm to 120 nm results in an incremental increase in the antitumour activity as evidenced by an increase in ILS from 27.8% to 77.8% at a vincristine dose of 1 mg/kg. Similar trends are observed for a vincristine dose of 2 mg/kg. This effect is most notable in DSPC/cholesterol preparations sized through 1.0- and 0.6-μm pore-size filters, where median survival times and long-term survival values are significantly reduced as compared with those obtained using the 120-nm systems ($P < 0.05$). That smaller liposomal vincristine preparations are more potent therapeutically is also reflected in the increased liposomal/free drug median survival time (L/F) ratios observed for these systems (Table 1). It should be noted, however, that the L/F values indicate that all DSPC/cholesterol-entrapped vincristine formulations display antitumour activity that is enhanced in comparison with that of the free drug.

The results presented in Table 2 demonstrate that the vesicle lipid composition can influence the antitumour activity of liposomal vincristine. Administration of vincristine encapsulated in EPC/cholesterol vesicles provides a slight increase in antitumour activity over that of the free drug, resulting in a maximal ILS value of 38.9% and L/F values as high as 1.16. Substitution of DSPC for EPC in the 120-nm vesicle systems results in a dramatic increase in the antitumour activity of liposomal vincristine. For example, the ILS value for vincristine given at a dose of 1 mg/kg increases from 5.5% for the free drug to 22.2% and 77.8% for EPC/cholesterol and DSPC/cholesterol formulations, respectively (Table 2). The L/F values of 1.68 and 1.83 observed for the DSPC/cholesterol system injected at drug doses of 1 and 2 mg/kg, respectively, indicate a significant increase in the antitumour potency of encapsulated vincristine over that of the free form of the drug ($P < 0.05$).

Another important characteristic of liposomal drug preparations is the drug-to-lipid ratio. This feature dictates the lipid dose required to deliver a given amount of drug. Manipulating the drug-to-lipid ratio of liposomal vincristine may modulate its antitumour activity, since alterations in the lipid dose have been shown to affect the pharmacological behaviour (circulation lifetime) of liposomes [1, 5, 6] as well as their ability to retain entrapped drug [24]. Reducing the drug-to-lipid ratio from 0.1 : 1 to 0.05 : 1 (w/w) for DSPC/cholesterol-encapsulated vincristine preparations (120-nm size) does not significantly alter the antitumour activity at a drug dose of 1 mg/kg (Table 3).

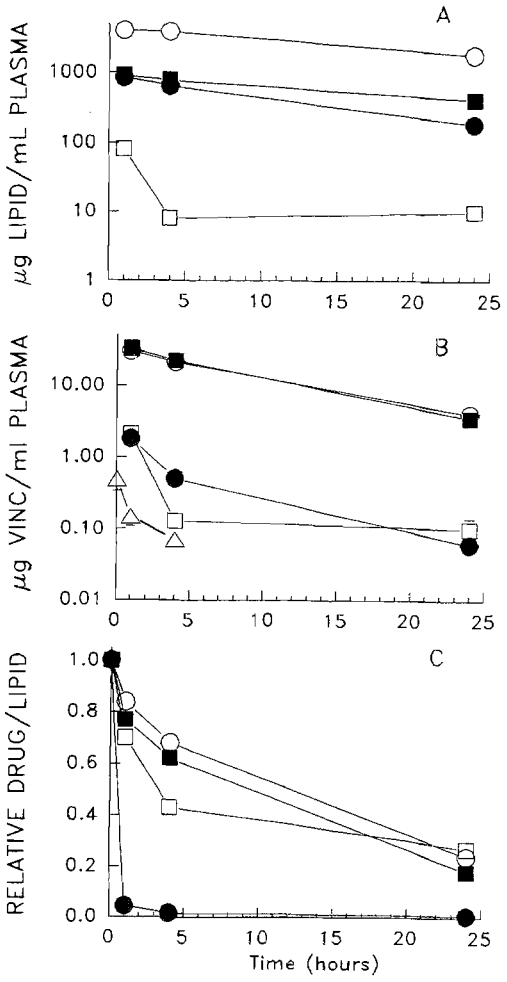

Fig. 3 A – C. Plasma concentrations of liposomal lipid (**A**) and vincristine (**B**) as well as the circulating drug-to-lipid ratio (**C**) in mice injected i.v. with free vincristine (△) or vincristine entrapped in 120-nm EPC/cholesterol (●), 1-μm DSPC/cholesterol (□) and 120-nm DSPC/cholesterol (○, ■) liposomes. The drug dose was 2 mg/kg and the drug-to-lipid ratio in the delivered liposomal systems was 0.05 : 1 (w/w) except for a 120-nm DSPC/cholesterol preparation that displayed a drug-to-lipid ratio of 0.01 : 1 (○). Error bars (present on all symbols) represent standard deviations calculated from four mice

However, a vincristine dose of 2 mg/kg given at a drug-to-lipid ratio of 0.05 : 1 (40 mg lipid/kg) appears more efficacious than preparations with a drug-to-lipid ratio of 0.1 : 1 (20 mg lipid/kg). This effect is related primarily to the increased rate of 60-day survival rather than to an increase in the median survival time (Table 3). Decreasing the drug-to-lipid ratio further to 0.01 : 1 (200 mg lipid/kg) results in a significant reduction in antitumour activity at a vincristine dose of 2 mg/kg. Injection of liposomal preparations exhibiting this lower drug-to-lipid ratio results in a decrease in the median survival time as well as the elimination of long-term survivors (Table 3).

On the basis of median survival times and long-term survival values, the present data thus far indicate that small (120-nm) vesicles composed of DSPC/cholesterol containing encapsulated vincristine at a drug-to-lipid ratio of 0.05 : 1 (w/w) display optimized antitumour potency as demonstrated by the survival curves presented in Fig. 2. Administration of liposomal vincristine at a dose of 2 mg/kg leads to a dramatic therapeutic effect as compared with that resulting from injection of the free drug at the same dose. In addition, the liposomal vincristine formulation can be given at doses higher than those tolerated for the free drug due to the decrease in toxicity provided by liposome encapsulation. Administration of liposomal vincristine at the elevated dose of 3 mg/kg results in further increases in the long-term survival of mice inoculated with L1210 cells (Fig. 2).

Pharmacological analysis of free and liposomal vincristine preparations

Previous investigations have shown that in addition to drug concentration, the cytotoxic activity of vincristine is very sensitive to the duration of cell exposure to the drug [18] and of drug retention by the cell [16]. Table 4 demonstrates the relationship between drug concentration and exposure time for the in vitro cytotoxicity of free vincristine against L1210 cells. The drug concentration required to yield 50% cytotoxicity (IC_{50}) decreases from 12 μ*M* to 0.12 n*M* as the duration of drug exposure is increased from 1 to 72 h. Given this extreme dependence of cytotoxicity on drug exposure time and the wide variations of drug leakage in blood observed among different liposomal systems (Fig. 1), the pharmacokinetic behaviour of liposomal vincristine formulations was investigated in an attempt to correlate antitumour activity with plasma clearance properties.

Figure 3 presents the plasma concentrations of liposomal lipid and vincristine measured over 24 h after i.v. injection of the free drug as well as vincristine entrapped in 120-nm EPC/cholesterol, 120-nm DSPC/cholesterol and 1-μm DSPC/cholesterol liposomes. The plasma lipid levels indicate that large (1-μm) DSPC/cholesterol liposomal vincristine preparations are rapidly cleared from the circulation, yielding lipid concentrations approximately 10-fold lower than those measured for the 120-nm systems at 1 h post-injection (Fig. 3A). For small (120-nm) vincristine-containing liposomes, substituting EPC for DSPC does not dramatically alter liposome clearance from the circulation within 4 h of i.v. injection. The lipid concentrations observed in the plasma at 24 h after injection are 420 μg/ml for the 120-nm DSPC/cholesterol system and 190 μg/ml for the 120-nm EPC/cholesterol preparation. These values represent total plasma liposome levels that amount to 47%

Table 4. Effect of exposure time on the in vitro cytotoxicity of vincristine against L1210 cells

Exposure time (h)[a]	IC_{50} (n*M*)[b]
1	12,000
4	2,400
6	2,400
24	7.3
72	0.12

[a] Duration of drug exposure starting at t = 0 over a total incubation period of 72 h

[b] Vincristine concentration required to achieve 50% cytotoxicity

and 21% of the delivered lipid dose, respectively. Decreasing the drug-to-lipid ratio of the 120-nm DSPC/cholesterol systems from 0.05 : 1 to 0.01 : 1 (w/w) increases the plasma lipid levels by approximately 5-fold, and 48% of the injected lipid dose remains in the circulation at 24 h after injection.

The plasma drug levels produced by these liposomal vincristine systems as well as by the free drug are shown in Fig. 3B. Administration of free vincristine results in very rapid removal of the drug from the circulation; 0.5 μg drug/ml plasma (which reflects 1% of the injected dose) is observed at 5 min after injection and no vincristine can be detected at the 24-h time point. Encapsulating vincristine in liposomes increase the circulating drug levels in a manner that is dependent on the physical properties of the vesicle carrier system. The plasma vincristine concentrations provided by 120-nm DSPC/cholesterol preparations decrease from approximately 30 to 3.5–3.9 μg/ml between 1 and 24 h post-injection. These values represent circulating plasma vincristine concentrations that correspond to 70% and 8% of the injected dose, respectively. It should be noted that HPLC analysis indicated that >90% of the radioactivity recovered in the plasma was due to intact vincristine. Increasing the vesicle size or changing the phospholipid component to EPC results in a significant decrease in plasma vincristine concentrations over the 24-h time course. In the case of the 1-μm DSPC/cholesterol formulation, the drug clearance from the plasma (Fig. 3B) correlates well with the removal of the liposomes (Fig. 3A), and the circulating drug-to-lipid ratios determined over the 24-h time course amount to >30% of the ratio calculated before in vivo administration (Fig. 3C). In contrast, the vincristine concentrations decrease rapidly for 120-nm EPC/cholesterol systems (Fig. 3B) even though the lipid levels remain relatively high over the 24-h time course (Fig. 3A). As a result, the drug-to-lipid ratio in plasma shows a 47-fold decrease within 1 h of injection (Fig. 3C). A comparison of the 120-nm DSPC/cholesterol systems injected at initial drug-to-lipid ratios of 0.05 : 1 and 0.01 : 1 reveals that the circulating drug-to-lipid ratios at 24 h are reduced by 83% and 76%, respectively.

Discussion

Vincristine is a potent antineoplastic agent that is commonly employed in cancer chemotherapy regimens. Initial murine studies have indicated that appropriately designed liposomes may improve the therapeutic index of this drug [26]. Specifically, vincristine encapsulated in DSPC/cholesterol vesicles using pH gradient-dependent entrapment procedures is more potent than the free drug with respect to antitumour activity and is also less toxic. The work presented herein extends on this observation to identify the roles of liposome characteristics that are known to affect the pharmacological behaviour of vesicles in amplifying the therapeutic efficacy of encapsulated vincristine.

Because the antitumour activity of vincristine is dependent on drug concentration and exposure time, vesicle physical properties that may potentially affect the pharmacokinetic behaviour of vincristine encapsulated in liposomes were evaluated in the present antitumour therapy and pharmacology studies. With regard to the clearance of liposomes from the circulation, previous investigations have shown that decreasing the vesicle size from >1 μm to <100 nm [3, 4, 17, 31] or increasing the lipid dose (which corresponds to a decrease in the drug-to-lipid ratio) [1, 5, 6] can increase liposome circulation lifetimes by 1 order of magnitude or more. Another important feature is the stability of the liposomal vincristine preparation, since rapid release of drug from the vesicle carrier may obviate any benefits provided by a long-circulating liposomal system. The present investigations therefore focused on liposomal vincristine formulations varying in size (from 100 nm to 1 μm), lipid dose (drug-to-lipid ratios ranging from 0.1 : 1 to 0.01 : 1, w/w) and drug retention (EPC/cholesterol versus DSPC/cholesterol). Because such variables can be readily controlled in generating liposomal vincristine formulations employing pH gradient-dependent encapsulation techniques, the relative importance of each characteristic in determining the biological activity of the formulation could be assessed individually.

Examination of the therapeutic activity and pharmacological behaviour of free vincristine provides the basis for understanding the increased efficacy observed for liposomal systems. The results presented herein suggest that the poor antitumour activity of free vincristine against the L1210 tumour model may be related to the rapid clearance of drug from the blood compartment. At 30 min post-injection, for example, approximately 99.8% of the injected vincristine dose has been removed from the circulation. The remaining plasma drug concentrations are several orders of magnitude lower than those required to induce cytotoxicity in vitro (cf. Table 4 and Fig. 3). Although a direct comparison between in vitro and in vivo drug concentrations may not be quantitatively valid, such relationships correlate well with the lack of significant in vivo antitumour activity observed for free vincristine in the present studies. These results also compare favourably with previous in vitro and in vivo experiments [14, 15, 18] that examined the concentration/exposure time relationship for the antitumour activity of vincristine.

Administration of vincristine in a liposome-encapsulated form (using pH gradient-derived systems) results in enhanced efficacy over that of the free drug for all systems investigated in the current studies. Furthermore, earlier studies [26] also indicate that such effects are obtained for a variety of multiple-dose regimens. However, dramatic differences in antitumour activity can be achieved by altering the vesicle physical properties mentioned above. Specifically, substituting DSPC for EPC or decreasing the size of DSPC/cholesterol vesicles from 1 μm to 120 nm results in a substantial increase in the antitumour potency of liposomal vincristine. This effect is manifested by a 2- to 4-fold increase in ILS values at drug doses of 1 and 2 mg/kg as well as the occurrence of long-term survival. The relative antitumour activities of these three preparations correlate well with their plasma drug concentration/time curves. The 120-nm DSPC/cholesterol preparation exhibiting a drug-to-lipid ratio of 0.05 : 1 (w/w) provides high plasma vincristine levels over the present 24-h

time course. In the case of EPC/cholesterol- and 1- μm DSPC/cholesterol-encapsulated vincristine, approximately 96% and >99% of the delivered dose is cleared from the circulation within 1 and 24 h of i.v. administration, respectively. For EPC/cholesterol systems, this effect results from leakage of vincristine from the vesicles and a corresponding 47-fold reduction in the circulating drug-to-lipid ratio. In contrast, the plasma drug-to-lipid ratio for 1-μm DSPC/cholesterol formulations remains relatively constant. Depletion of vincristine from the plasma for this preparation occurs primarily by clearance of drug-containing liposomes by the phagocytic cells of the liver and spleen. The above-mentioned results confirm predictions based on the behaviour of the free drug and indicate that the antitumour efficacy of liposomal vincristine is closely related to the longevity of the drug in the blood compartment. These results are also consistent with observations made in studies investigating vincristine infusion. Clinical trials of vincristine infused over 5 days yielded tumour responses in patients with refractory malignancies, some of which had previously been treated with vincristine given as a bolus [19, 20].

The increased antitumour activity reported herein for small liposomal vincristine systems agrees with the results of previous investigations into the effects of vesicle properties on the antitumour efficacy of liposomal doxorubicin [23]. In addition to the capacity of small liposomes to provide a long-circulating slow-release depot of free drug. their enhanced antitumour activity may arise from their ability to access extravascular sites unavailable to larger systems [9, 11, 25, 29]. In this context, it should be noted that pharmacokinetic experiments in the present study were not capable of distinguishing between unentrapped and vesicle-associated forms of intravenously injected liposomal vincristine. However, a comparison of the antitumour activity and pharmacokinetic behaviour of small (mean diameter, approximately 120 nm) systems suggest that time-sustained release of encapsulated drug from the vesicles into the circulation or into accessible extravascular sites exposed to tumour cells may be an important factor in determining the therapeutic efficacy of liposomal vincristine. For example, at 24 h after the injection of small EPC/cholesterol and DSPC/cholesterol (drug-to-lipid ratio, 0.05 : 1) liposomal vincristine preparations, the drug-to-lipid ratios of the circulating vesicles are 1% and 17% of the respective initial values. In the case of EPC/cholesterol, the antitumour efficacy is inferior to that of the 120-nm DSPC/cholesterol system displaying a drug-to-lipid ratio of 0.05 : 1 (w/w). Taken together, the results implicate the necessity of a circulating liposomal vincristine reservoir that can provide sufficient therapeutic free drug levels over extended periods so as to enhance antitumour therapy. The ability of small liposomes to deliver their contents directly to tumour sites, however, cannot be dismissed as a contributing factor to this phenomenon. This is a potential explanation for the observation that although DSPC/cholesterol liposomal vincristine preparations with a drug-to-lipid ratio of 0.01 : 1 (w/w) exhibit improved circulation longevity, they display a modest decrease in antitumour activity as compared with that observed for formulations with a 0.05 : 1 drug-to-lipid ratio.

The relationship between vincristine's longevity in the circulation and its efficacy is of particular interest in the context of the design of liposomal vincristine formulations with improved therapeutic activity. Specifically, although increased liposome circulation lifetimes may be obtainable by the use of GM-1- or PEG-derivatized lipids [2, 8, 9, 11], the benefit of such properties may be limited by the ability of vincristine to permeate readily even rather "rigid" bilayer membranes such as the DSPC/cholesterol systems examined in the present study. Consequently, it is likely that further improvements in the antitumour activity of liposomal vincristine beyond that observed for 120-nm DSPC/cholesterol preparations will rely more on enhanced drug retention in the liposomes than on increased circulation longevity of the vesicle systems themselves.

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