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ORIGINAL ARTICLE

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Preclinical pharmacology, toxicology and efficacy of sphingomyelin/cholesterol liposomal vincristine for therapeutic treatment of cancer

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Abstract *Purpose*: To establish the pharmacodynamic relationships between drug biodistribution and drug toxicity/efficacy, a comprehensive preclinical evaluation of sphingomyelin/cholesterol (SM/chol) liposomal vincristine and unencapsulated vincristine in mice was undertaken. *Methods*: Pharmaceutically acceptable formulations of unencapsulated vincristine and liposomal vincristine at drug/lipid ratios of 0.05 or 0.10 (wt/wt) were evaluated for toxicity, antitumor activity and pharmacokinetics following intravenous administration. *Results*: Mice given liposomal vincristine at 2 mg/kg vincristine had concentrations of vincristine in blood and plasma at least two orders of magnitude greater then those achieved after an identical dose of unencapsulated drug. One day after administration of the liposomal vincristine, there were at least tenfold greater drug quantities, relative to unencapsulated vincristine, in the axillary lymph nodes, heart, inguinal lymph nodes, kidney, liver, skin, small intestines and spleen. Increased plasma and tissue exposure to vincristine as a result of encapsulation in SM/chol liposomes was not associated with increased drug toxicities. Treatment of the murine P388 ascitic tumor with a single intravenous dose of unencapsulated drug at 2, 3 and 4 mg/kg, initiated 1 day after tumor cell inoculation, resulted in a 33 to 38% increase in lifespan. In contrast, long-term survival rates of 50% or more were achieved in all groups treated with the SM/chol liposomal vincristine formulations at doses of 2, 3 and 4 mg/kg. At the 4 mg/kg dose, eight of ten and nine of ten animals survived past day 60 when treated with SM/chol liposomal vincristine prepared at the 0.05 and 0.1 drug/lipid ratios, respectively. *Conclusions*: Overall, increased and prolonged plasma concentrations of vincristine achieved by liposomal encapsulation were correlated with dramatically increased antitumor activity in comparison with the unencapsulated drug, but no correlations could be established between pharmacokinetic parameters and toxicity.

Key words Vincristine · Liposome · Pharmacology

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Introduction

Preclinical studies in mice have demonstrated that vincristine encapsulated in distearoylphosphatidylcholine/cholesterol (DSPC/chol) liposomes exhibits significantly enhanced circulation longevity and antitumor efficacy compared to unencapsulated vincristine [6, 9, 10]. These properties, in addition to the potentially reduced neurotoxicity for a liposome formulation, provided the basis for pursuing a phase I clinical trial of this formulation at the British Columbia Cancer Agency [11]. While the DSPC/chol formulation of vincristine has significant therapeutic potential, its pharmaceutical utility is compromised by stability issues. This formulation utilizes a pH gradient drug encapsulation procedure where the external medium of liposomes prepared at pH 4.0 is alkalinized to pH 7.5 and vincristine is quantitatively entrapped inside the liposomes just prior to use [9]. Consequently, the liposomes must be prepared and stored at pH 4.0. The acyl chains of DSPC, however, are ester linked and subject to acid catalyzed hydrolysis [15]. The resulting degradation products give rise to increased vincristine leakage from the liposomes after intravenous injection. For this reason the DSPC/chol formulation of liposomal vincristine has a shelf-life of between 9 and 12 months during storage at 4 °C.

A substantial reduction of the degradation of the lipid component would significantly improve the pharmaceutical acceptability of liposomal vincristine. Since the aliphatic chain of sphingomyelin (SM), an abundant sphingolipid present in animal tissues, is amide linked rather than ester linked, SM is significantly less susceptible to acid hydrolysis than is DSPC [15]. Furthermore, because the headgroup moiety of SM is identical to that of DSPC, it could be expected that liposomes composed of SM and cholesterol (chol) would have circulation and drug retention characteristics similar to those of the DSPC/chol formulation. Surprisingly, initial preclinical studies have indicated that the SM/chol formulation is therapeutically more active in preclinical tumor models than the DSPC/chol formulation of vincristine [15]. This improved therapy was a consequence of improved drug retention characteristics of the SM/chol formulation [15], a conclusion that is supported by the results of Boman et al. [4].

It was the intention of this effort to identify a liposomal vincristine formulation with an optimized therapeutic index for evaluation in phase I/II clinical trials and correlate therapeutic activity with specific pharmacokinetic properties. For this purpose, vincristine was encapsulated in SM/chol liposomes that were prepared under current Good Manufacturing Practice conditions (cGMP, according to CFR 21, part 211) at drug/lipid ratios of 0.05 and 0.10 and compared with unencapsulated drug in toxicity, efficacy and pharmacokinetic studies. The selection of two drug/lipid ratios was intended to determine whether (1) the higher lipid dose conferred by the lower drug/lipid ratio would lead to alterations in the rate of lipid and/or drug clearance [8], and (2) improved drug retention would be observed at the lower drug/lipid ratio, as previously described for doxorubicin [8]. These investigations provided greater insight into the role that liposome-associated alterations in drug disposition play in dictating the toxicity and efficacy of vincristine.

Materials and methods

Materials

SM/chol liposomes (100 nm) were obtained from the British Columbia Cancer Agency Investigational Drug Section (IDS) (Lot #IS001) and prepared as outlined below. These liposomes were prepared from cGMP-grade sources for egg SM (Avanti Polar Lipids, Alabaster, Alabama), chol (Croda, Parsippany, NJ), citrate, sodium citrate, sodium phosphate and sodium chloride from J.T. Baker, Phillipsburg, NJ. Water For Injection and saline (0.9% Sodium Chloride Injection USP) from Baxter, Toronto, ON. Vincristine Sulfate Injection (Oncovin, as a 1 mg/ml solution) was obtained from Eli Lilly, Toronto, ON, Canada, and [^{3}H]-vincristine sulfate obtained from Amersham, Oakville, ON, Canada. P388 cells were obtained from the NCI tumor repository.

Animals

Female BDF1 mice (*Mus musculus*) 8–12 weeks old were obtained from Charles River, St. Constant, Quebec, Canada, and were housed in conventional cages (Nalgene, Rochester, NY) on wood shavings. The rooms were maintained on a 12-h light/dark cycle with a temperature range between 19 and 25 °C and humidity range between 30 and 70%. The animals were fed standard commercial mouse food (LabDiet, The Richmond Standard) and municipal tapwater, ad libitum. Animals weighing between 19 and 21 g were selected for use 4 days prior to initiation of the study. Male and female CR:NIH(S)-F mice (4–5 weeks old) were obtained from the Frederick Cancer Research and Development Center, National Cancer Institute, Frederick, Md.

Liposome preparation and vincristine encapsulation

The liposomes and sodium phosphate solution used for these studies were prepared under cGMP conditions according to the procedures specified in Master Batch Record Reference # 102-01-REV.1 maintained at the B.C. Cancer Agency IDS. Briefly, these liposomes were composed of 72 wt% SM and 28 wt% chol (representing 55 mol% SM and 45 mol% chol) and hydrated using a 300 m*M* citrate buffer (pH 4.0) to achieve a final lipid concentration of 100 mg/ml. Lipid hydration was facilitated by three freeze and thaw cycles. The liposomes were prepared by extruding the hydrated lipid through polycarbonate filters (Nuclepore, Pleasanton, CA) using an 800-ml Thermobarrel Extruder (Lipex Biomembranes, Vancouver, Canada). The resulting unilamellar liposomes had a mean diameter (assessed by quasielastic light scattering) of 128 nm. The liposomes were maintained at 4 °C prior to use.

Vincristine for encapsulation in SM/chol liposomes, or for administration of unencapsulated vincristine, was prepared by mixing Vincristine Sulfate Injection with 10 μCi radiolabelled [^{3}H]-vincristine sulfate per mg of unlabelled vincristine. Vincristine was encapsulated in SM/chol liposomes using the pH-gradient loading technique [6, 9, 10]. Radiolabelled vincristine was added to preformed SM/chol liposomes (pH 4.0) to achieve final drug/lipid ratios of either 0.05 or 0.10 (wt/wt), then the external pH was increased to 7.3 by the addition of 0.1 *M* sodium phosphate buffer (IDS Lot # IC005). The mixture was then incubated at 63 °C for 10 min during which time vincristine was encapsulated in the liposomes. The resulting solutions were cooled to room temperature prior to any further dilutions required for dosing the mice (see below). Trapping efficiencies of greater than 95% are routinely achieved using this procedure [9, 10].

Pharmacokinetics and biodistribution experiments

Unencapsulated vincristine and liposomal vincristine, radiolabelled with [^{3}H]-vincristine as described above, were prepared at a final concentration of 0.16 mg/ml by dilutions using 0.9% Sodium Chloride Injection USP. Mice were injected at a 2 mg/kg drug dose (approximately 0.4 μCi per mouse) in a volume of 250 μl as a single bolus intravenous injection via the lateral tail vein. The dose was estimated on the basis of a mean body weight of 20 g. This drug dose corresponded to a lipid dose of either 40 mg/kg or 20 mg/kg for the liposomal formulations at drug/lipid ratios of 0.05 or 0.10 (wt/wt), respectively.

At various times after administration, mice were euthanized by CO_2 asphyxiation, blood was removed by cardiac puncture and placed into EDTA-coated microtainers (Becton Dickinson) on ice. Tissues from the animals were removed, washed in phosphate-buffered saline and weighed prior to storing the tissue at −70 °C for at least 12 h. The tissues removed included lung, heart, spleen, liver, kidney, stomach, small intestine, large intestine, muscle, skin, brain, inguinal and axillary lymph nodes as well as bone marrow (whole femur).

Blood, plasma and tissue analysis

A 50-μl aliquot of whole blood was removed and processed for scintillation counting as described below for the tissue samples. The

remaining sample was centrifuged at 500 *g* in a refrigerated (approximately 4 °C) clinical bench-top centrifuge. Plasma was carefully pipetted away from the pelleted cells and placed into an Eppendorf tube (maintained on ice). A 50-µl aliquot of plasma was removed and assayed for [^{3}H]-vincristine concentrations by scintillation counting. From the remaining plasma, 100 µl was transferred to a Microcon-30 (Amicon, Nepean, Ontario) device and centrifuged at 4 °C for 10 min at 14 000 *g*. A 50-µl aliquot of the ultrafiltrate was then assayed for [^{3}H]-vincristine by scintillation counting.

Where indicated, thawed tissue samples were homogenized in distilled water to achieve a final 10% homogenate (w/v). Aliquots (200 µl) of the homogenates were mixed with 0.5 ml of Solvable (NEN, Du Pont Canada, Mississauga, ON) and incubated at approximately 50 °C for a period of 3 h. Tissues that could not be readily homogenized (skin, femur) and tissues that weighed less then 50 mg (lymph nodes) were digested directly in Solvable. Subsequently, the samples were cooled to room temperature and then 50 µl of 200 m*M* EDTA was added followed by 200 µl of a 30% solution of H_2O_2 and 25 µl of 10 *N* HCl. After the samples had been left for 1 h at room temperature, 5 ml of PicoFluor Scintillation fluid was added and the tissue concentrations of vincristine determined by scintillation counting.

Levels of drug in the liver, spleen, lung, kidney, muscle, heart and brain were corrected for tissue plasma volumes [12]. Plasma volumes for other tissues (stomach, small intestine, large intestine, skin, inguinal nodes, axillary nodes and bone marrow or whole femur) were not estimated, so that the reported values do not account for levels in the blood compartment of the tissue. Free drug levels in the plasma of animals receiving liposomal vincristine were based on radioactivity found in the filtrate of plasma samples after separation using the Microcon 30 device [7]. It should be noted that the amount of [^{3}H]-vincristine measured in tissues and blood due to intact vincristine was not distinguished from vincristine metabolites.

Acute toxicity model

A single-dose acute lethality and toxicity study was performed in CR:NIH(S)-F mice after intravenous administration of unencapsulated vincristine, vincristine encapsulated in SM/chol liposomes (drug/lipid ratio 0.05) or empty SM/chol liposomes, or in no-treatment controls. Five male and female mice per dosage group were given unencapsulated vincristine and liposomal vincristine at 2, 4 or 6 mg/kg then monitored daily for 37 days for individual body weights, clinical signs and lethality. Empty SM/chol liposomes were administered at the lipid dose equivalent to that given at the 6 mg/kg vincristine dose for encapsulated drug then monitored as described above.

P388 antitumor efficacy model

Female BDF1 mice in 12 groups (see Table 3), five mice per group in each of two experiments were injected (intraperitoneally) with 10^6 P388 cells. One day after tumor cell inoculation the mice were given a bolus intravenous injection of unencapsulated vincristine, 0.05 drug/lipid ratio SM/chol liposomal vincristine or 0.10 drug/lipid ratio SM/chol liposomal vincristine at doses of 2, 3 or 4 mg/kg via the lateral tail vein. Control groups were injected with either saline or empty liposomes (IDS lot # IS001) with the treatment groups organized as described in Table 1. Following administration of the test materials, animals were weighed daily for 14 days and monitored for signs of stress twice daily for the first 14 days and once daily for the remainder of the study. Survival time was recorded for animals in each group. Animals surviving longer than 60 days were considered long-term survivors.

Statistical analysis of the data

The efficacy data were evaluated using a survival analysis with censored regression. This analysis was used since the study was

Table 1 Summary of the pharmacodynamic parameters of unencapsulated vincristine and SM/chol liposomal vincristine

Tissue	Unencapsulated vincristine		SM/chol vincristine (0.05)		SM/chol vincristine (0.10)	
	C_{max} (µg/g)	AUC_{0-24} (µg/g/h)	C_{max} (µg/g)	AUC_{0-72} (µg/g/h)	C_{max} (µg/g)	AUC_{0-72} (µg/g/h)
Category 1[a]						
Heart	0.538	1.98	0.723	24.3	0.876	27.9
Lung	0.290	3.25	1.18	26.9	1.65	23.3
Plasma[b]	0.06	0.70	27.5	228.4	29.3	211.3
Whole blood[b]	0.14	1.68	15.0	103.9	14.3	97.2
Category 2[a]						
Liver	0.832	3.82	2.20	65.0	2.30	56.5
Small intestines	1.43	6.48	1.75	62.0	2.08	65.5
Category 3[a]						
Axillary Node	0.740	3.23	1.73	71.4	2.20	82.5
Inguinal Node	0.831	2.40	1.85	71.5	2.90	90.6
Kidney	0.982	2.50	1.58	75.1	1.69	76.2
Large intestines	1.38	5.06	0.748	31.0	0.859	33.0
Skin	0.251	1.02	0.611	24.3	0.648	23.1
Spleen	1.69	14.7	8.43	407.4	8.27	420.4
Stomach	0.621	2.89	0.691	27.7	0.597	24.0
Category 4[a]						
Bone Marrow[c]	0.022	0.14	0.143	4.21	0.031	1.00
Brain	0.218	0.24	0.027	0.94	0.018	0.86
Muscle	0.547	2.80	0.097	3.31	0.107	5.00

[a] C_{max} at 1 h, 4 h, and 24 h postadministration for the tissues in groups 1, 2 and 3 respectively. Group 4 tissue levels of drug were transiently higher after administration of unencapsulated vs liposomal vincristine or the tissue quantities of vincristine were negligible

[b] Plasma and whole blood values converted from µg/ml

[c] bone marrow values expressed in µg/femur

given an arbitrary time limit (60 days) and several of the treatment groups contained long-term survivors. Different treatment groups were compared using a two-sample Cox's *f*-test. It should be noted that the assumptions used in this analysis require a minimum of three uncensored samples (a minimum of three tumor-related deaths). Those groups where eight or more animals survived could not be analyzed via Cox's *f*-test. For these groups, survival times were estimated based on the assumption that the surviving animals died on day 61. Survival data were expressed as mean survival time (days), median survival time (days), percentage increase in lifespan (median survival time of treated groups divided by the median survival time of untreated groups minus 1 and multiplied by 100) and the number of long-term survivors (no. of survivors/*n*).

Results

Pharmacokinetics

The concentrations of vincristine in blood following intravenous administration of unencapsulated drug and the two different formulations of SM/chol liposomal vincristine are shown in Fig. 1A. Consistent with previous observations [15], the level of drug in blood of animals receiving liposomal drug was greater than that observed following administration of unencapsulated drug. By 1 h after drug administration less than 0.8% of the injected unencapsulated drug was recovered in blood, compared with 75% and 72% of injected drug recovered after administration of SM/chol liposomal vincristine (0.05 drug/lipid and 0.10 drug/lipid ratios, respectively). There were no significant differences between the blood concentrations of vincristine in animals receiving SM/chol liposomal vincristine at the two drug/lipid ratios up to 72 h after administration (Fig. 1A).

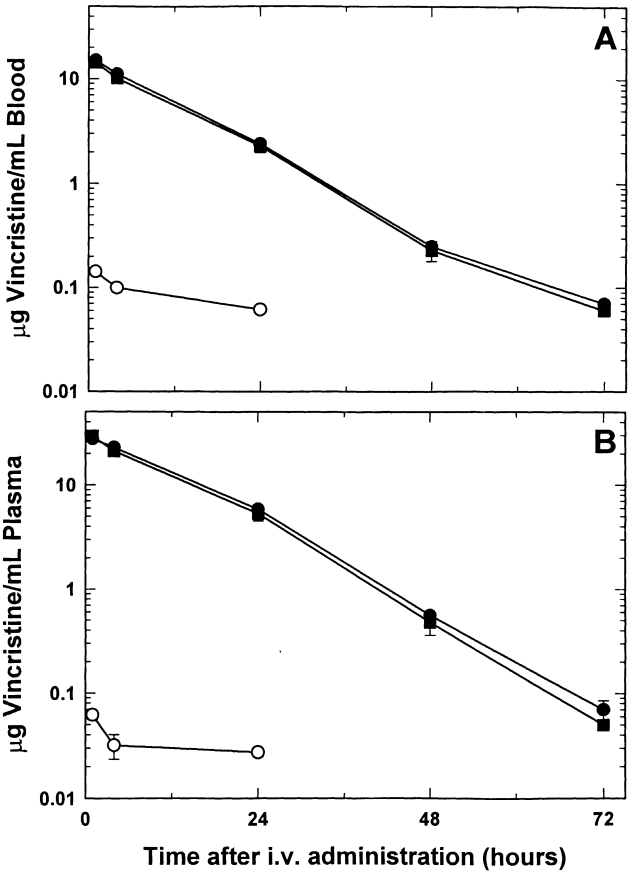

Fig. 1A,B Vincristine levels in whole blood (**A**) and plasma (**B**) obtained from BDF1 mice after intravenous administration of unencapsulated vincristine (O) or vincristine encapsulated in SM/chol liposomes at final drug/lipid ratios of either 0.05 (●) or 0.10 (■). The data points represent the mean values (± standard error) from four mice; where error bars are not visible, they are smaller than the size of the symbol

Analysis of plasma confirmed that significant increases in drug concentrations in the circulation occurred as a consequence of encapsulation of vincristine in SM/chol liposomes (Fig. 1B). As observed in blood (Fig. 1A), there were no differences between the two liposomal formulations. The circulation half-life for vincristine in the plasma was increased from < 0.5 h for the unencapsulated drug to 8.2 and 7.8 h for the SM/chol liposomal formulations prepared at drug/lipid ratios of 0.05 and 0.10, respectively. The increases in circulating concentrations of vincristine and the circulation lifetimes of vincristine due to encapsulation were reflected in significant increases in the area under the curve (AUC) values for vincristine (Table 1) and 60- and 300-fold increases in the ratio of $AUC_{liposomal}/AUC_{unencapsulated}$ in blood and plasma, respectively (Table 2).

The clearance curves presented in Fig. 1 suggest a single elimination phase for the liposomal vincristine, with no apparent distribution phase, compared to a very rapid and significant distribution phase, followed by one or two slower elimination phases for the unencapsulated drug. It should be noted that the ratio of blood vincristine concentrations versus plasma vincristine concentrations in animals that received liposomal vincristine averaged 0.466. Since the systemic hematocrit of BDF1 mice has been reported to be 46% [12], this suggests that the majority of drug in the blood from animals given liposomal vincristine was non-cell-associated. A similar analysis of blood/plasma vincristine ratios in animals given the unencapsulated drug gave an average of more than 2, suggesting that the majority of unencapsulated drug in the blood was cell-associated. This is consistent with the suggestion that platelets in blood can bind significant quantities of vincristine [5, 13].

Since encapsulation significantly increased blood and plasma concentrations of vincristine, it was necessary to determine if the circulating drug was encapsulated in liposomes or was present as free drug. It was anticipated that any drug present in the blood compartment, but remaining encapsulated in liposomes, would not be bioavailable to cause increases in toxicity and/or antitumor activity. To determine the level of free drug in the plasma of animals given liposomal vincristine, plasma was filtered through a 30 000 MWCO filter using a Microcon-30 device. This device separates free vincristine and vincristine bound to proteins of less than

30 000 Da (collected in the filtrate) from vincristine bound to larger proteins and encapsulated in liposomes. Free drug levels in the plasma of animals treated with the liposomal formulations were below detectable limits (0.05 μg/ml) at 1, 4 and 24 h after administration. A comparison of the free drug concentrations in the plasma with the total drug concentrations in the plasma (Fig. 1B) in animals treated with liposomal vincristine indicated that less than 1% of the drug present in the plasma was present as free vincristine, regardless of the drug/lipid ratio used (data not shown). That is, >99% of the vincristine in the plasma was encapsulated within the SM/chol liposomes.

Biodistribution of vincristine

The biodistribution of unencapsulated vincristine and SM/chol liposomal vincristine after intravenous administration is summarized in Fig. 2. We classified the examined tissues into four different categories based on the disposition of liposomal vincristine. In category 1, the tissue C_{max} for the drug was observed at 1 h postadministration and decreased at later times. Tissues described by this behavior include the lung (Fig. 2A) and the heart (data not shown) as well as both whole blood and plasma (Fig. 1). Category 2 tissues were characterized by a C_{max} occurring at or around 4 h postadministration and included the liver (Fig. 2B) and small intestines (data not shown). In category 3 tissues, the quantities of vincristine increased to the C_{max} at 24 h postadministration then decreased at later times. Tissues in this category included the spleen (Fig. 2C), kidney, skin and axillary node (data not shown). We also included the inguinal node, large intestines and stomach in this categrory even though the vincristine quantities in these tissues were very similar at 4 and 24 h postadministration. Finally, category 4 included those tissues, such as muscle (Fig. 2D) and bone marrow (data not shown), in which the levels of drug were transiently higher after administration of unencapsulated vincristine than after liposomal vincristine (typically at 1 h postadministration; Fig. 2D) or the quantities of vincristine in the tissue were negligible (brain; data not shown).

The relationships between the unencapsulated vincristine and liposomal treatments and the various tissues are quantitatively summarized in Table 1. Encapsulation of vincristine in SM/chol liposomes resulted in increased drug exposure for all tissues examined compared to unencapsulated vincristine, with the exception of muscle (Tables 1 and 2). This is demonstrated by: (1) increased C_{max} values in the tissues of category 1–3 after vincristine encapsulation, (2) increased ratios of vincristine recovered after liposomal treatment to vincristine recovered after unencapsulated drug administration (Table 2), and (3) increased AUC values (Table 1), and $AUC_{liposomal}/AUC_{unencapsulated}$ ratios (Table 2), after encapsulation. For example, at 24 h after administration, the ratios of drug recovered in tissues following administration of SM/chol liposomal vincristine (0.05 drug/lipid ratio) divided by drug recovered in tissues

Table 2 Summary of the ratios of vincristine recovered in various tissues after administration of SM/chol liposomes (at drug/lipid ratios of either 0.05 or 0.10) to that recovered in various tissues after administration of unencapsulated vincristine and the area under the curve (AUC) analysis of the total tissue exposure to liposomal/unencapsulated vincristine. The vincristine dose was 2 mg/kg for all treatments. Data are the mean values obtained from four animals

Tissue	SM/chol vincristine (0.05)/unencapsulated vincristine				SM/chol vincristine (0.10)/unencapsulated vincristine			
	1 h	4 h	24 h	$AUC_{liposomal}/AUC_{unencapsulated}$	1 h	4 h	24 h	$AUC_{liposomal}/AUC_{unencapsulated}$
Category 1								
Heart	1.34	2.05	11.3	12.3	1.63	1.92	25.5	14.1
Lung	4.01	8.73	4.22	8.28	5.69	9.37	3.44	7.17
Plasma	443.1	715.4	215.0	326.3	472.1	659.6	194.5	301.8
Whole blood	105.2	111.3	38.7	61.8	99.9	101.4	36.9	57.8
Category 2								
Liver	1.34	6.97	15.9	17.0	1.55	7.28	11.9	14.8
Small intestine	1.10	2.32	11.0	9.57	1.32	2.77	12.0	10.1
Category 3								
Axillary node	0.85	3.20	26.7	22.1	0.82	3.17	34.0	25.5
Inguinal node	0.68	4.93	78.0	29.8	0.68	3.00	133.2	37.7
Kidney	0.65	2.53	58.9	30.0	0.79	3.85	62.9	30.5
Large intestine	0.26	1.33	6.70	6.12	0.35	1.64	7.69	6.52
Skin	0.88	2.76	38.9	23.8	0.75	3.74	41.3	22.6
Spleen	1.98	4.14	18.5	27.7	0.49	3.21	18.1	28.6
Stomach	0.81	1.91	10.7	9.58	0.82	1.67	9.23	8.31
Category 4								
Bone marrow	1.26	2.26	6.97	30.5	1.42	2.51	6.00	7.22
Brain	0	0	–	3.82	0	0	–	3.53
Muscle	0	0.29	1.43	1.18	0.04	0.32	1.70	1.79

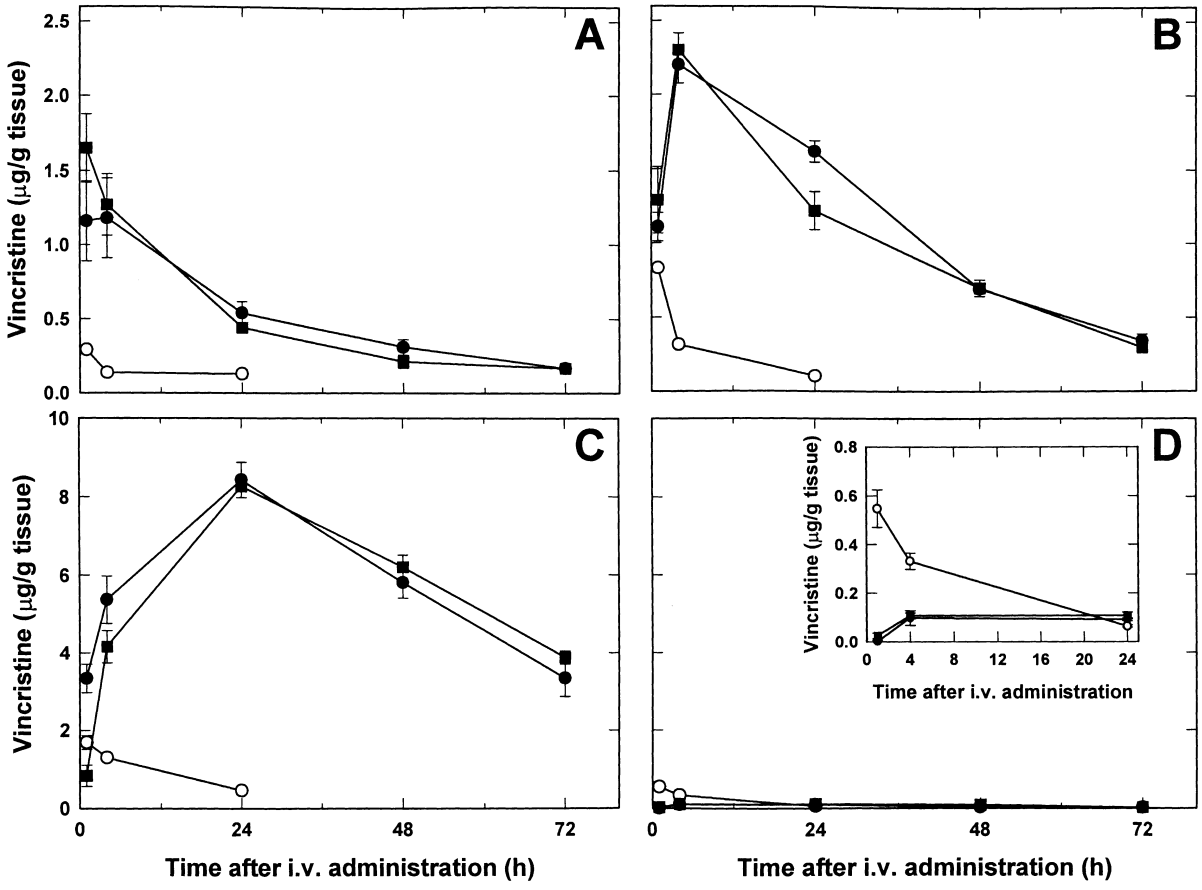

Fig. 2A–D Quantities of vincristine recovered in representative tissues. Vincristine was quantified by liquid scintillation counting after the intravenous administration of unencapsulated vincristine (O) or vineristine encapsulated in SM/chol liposomes at final drug/lipid (wt/wt) ratios of 0.05 (●) or 0.10 (■). Tissues represented are the lung (**A**), liver (**B**), spleen (**C**) and muscle (**D**). The data for muscle (**D**) are plotted using two different *y*-axis ranges to demonstrate the relationship with the other tissues (**A–C**) and the within-tissue data (**D**, *inset*). The data points represent the means (± standard error) from four mice; where error bars are not visible, they are smaller than the size of the symbol

following administration of unencapsulated drug were 15.9 for liver, 11.0 for small intestines, 10.7 for stomach, 6.7 for large intestines, 18.5 for spleen, 26.7 for axillary nodes, 78.0 for inguinal nodes, 58.9 for kidney and 38.9 for skin (Table 2). Similarly, the total tissue exposure to drug, as indicated by the $AUC_{liposomal}/AUC_{unencapsulated}$ ratio, increased by 6.1 (large intestines) to 30 (inguinal node, kidney and bone marrow) times as a consequence of encapsulation (Table 2). As observed in the blood compartment, there were no differences observed in the tissue levels of vincristine (C_{max}) or AUC values obtained from animals treated with the SM/chol liposomal formulations of vincristine at either 0.05 or 0.10 drug/lipid ratios (Tables 1 and 2).

The total recovery of drug from animals given SM/chol liposomal vincristine formulations was 14-, 17- and 30-fold greater than that obtained from animals treated with unencapsulated drug at 1, 4 and 24 h, respectively (data not shown). No significant differences were observed in the recovery data obtained between animals given the SM/chol liposomal formulations at either 0.05 or 0.10 drug/lipid ratios. At 24, 48 and 72 h after injection of SM/chol liposomal vincristine formulations, more than 22%, 6% and 2% (respectively) of the injected drug was recovered in the blood and tissues analyzed. Of this, the majority was recovered from the liver and spleen. In contrast, less than 1% of the total injected unencapsulated vincristine could be recovered in the blood and tissues at 24 h after administration (data not shown).

P388 antitumor efficacy

The data presented in Tables 1 and 2 demonstrate dramatic increases in the concentrations of vincristine in the blood compartment and altered tissue distribution as a consequence of encapsulation in SM/chol liposomes. It was expected that this increase in drug exposure may have been associated with increased antitumor activity if the liposomes extravasated from the circulation to tumor sites [15]. Survival data for mice bearing ascitic P388 tumors and treated with control treatments or either unencapsulated vincristine or liposomal vincristine are summarized in Table 3 and Fig. 3. Control groups treated with empty liposomes, at either the 40 mg/kg or the 80 mg/kg lipid dose, exhibited no improvement in lifespan relative to saline-treated controls. Further, the

Table 3 Antitumor activity of unencapsulated vincristine and liposomal vincristine. Unencapsulated vincristine and SM/chol liposomal vincristine were administered intravenously to BDF1 mice 24 h after intraperitoneal inoculation with 10^6 tumor cells. The lipid dose was determined from the vincristine/lipid ratio and the administered vincristine dose (*NS* not significant)

Group	Sample	fDose (mg/kg)		Survival data				*P*-value[a]
		Vincristine	Lipid	60-day survivors	Mean (days)	Median (days)	Increase in lifespan (%)	
1	Saline	–	–	0/10	12.1	12.0	–	
2	Unencapsulated vincristine	2	–	0/10	17.1	16.0	33	
3	Unencapsulated vincristine	3	–	0/10	17.1	16.5	38	
4	Unencapsulated vincristine	4	–	0/10	18.6	16.5	38	
5	SM/chol vincristine (0.05)	2	40	5/10	50.5	60.5	>400	0.0038
6	SM/chol vincristine (0.05)	3	60	7/10	54.7	61.0	>400	0.0002
7	SM/chol vincristine (0.05)	4	80	8/10	53.5	61.0	>400	0.0112
8	SM/chol vincristine (0.10)	2	20	6/10	51.3	61.0	>400	0.00013
9	SM/chol vincristine (0.10)	3	30	6/10	53.0	61.0	>400	0.0001
10	SM/chol vincristine (0.10)	4	40	9/10	58.4	61.0	>400	0.00689
11	SM/chol empty	–	80	0/10	11.4	11.0	−8	NS
12	SM/chol empty	–	40	0/10	11.2	11.0	−8	NS

[a] *P*-value derived from the Cox's *f*-test comparing survival following administration of unencapsulated vincristine and survival following administration of the liposomal vincristine at the identical drug dose

results demonstrated that the P388 antitumor activity of SM/chol liposomal vincristine (regardless of the drug/lipid ratio employed) was significantly better than unencapsulated drug at all doses, as determined by Cox's *f*-test (Table 3).

In every group treated with the liposomal drug, there were at least 50% long-term survivors (i.e. animals surviving beyond day 60). The median survival time of animals succumbing to the tumor increased from 16–16.5 days with unencapsulated vincristine treatment to more than 50 days for all liposomal vincristine treatments, representing an increase in lifespan of >400%. It should be noted that unencapsulated vincristine at the doses tested resulted in a statistically significant increase in lifespan of 33–38% in comparison with no-treatment controls. It should also be noted that there were no statistically significant differences (Cox's *f*-test) between SM/chol liposomal vincristine at a drug/lipid ratio of 0.05 and that at a ratio of 0.10 administered at an identical dose (data not shown). For both liposomal and unencapsulated drug there was no indication of a significant dose response curve over the dose range of 2 to 4 mg/kg evaluated in this study (Fig. 3).

Acute toxicity of unencapsulated vincristine and liposomal vincristine

The data presented in Tables 1 and 2 clearly demonstrate significant increases in tissue exposure to vincristine as a consequence of liposomal encapsulation. While the increase in exposure in blood and plasma over the 72 h after administration due to encapsulation was 60- and 300-fold, respectively, the increases in tissue levels were typically between 10- and 30-fold (Table 2). These increases in tissue exposure to vincristine were significant and it was expected, therefore, that these increases may have been associated with increased toxicities. A comprehensive toxicity study using male and female CR:NIH(S)-F was performed using SM/chol liposomal vincristine. The 0.05 vincristine/lipid ratio was chosen for this study because there were no pharmacokinetic (Fig. 1), biodistribution (Fig. 2, Tables 1 and 2) or antitumor activity (Fig. 3, Table 3) differences between this formulation and that prepared using a drug/lipid ratio of 0.10. Further, we believed that the liposomes prepared at a 0.05 drug/lipid ratio would be a more stable product and, consequently, performed these toxicity studies with this formulation.

A slight reversible weight loss of 8.3% to 9.5% was observed after treatment of CR:NIH(S)-F mice with unencapsulated vincristine at 2.0 mg/kg but 100% lethality occurred at doses of 4.0 and 6.0 mg/kg (Table 4).

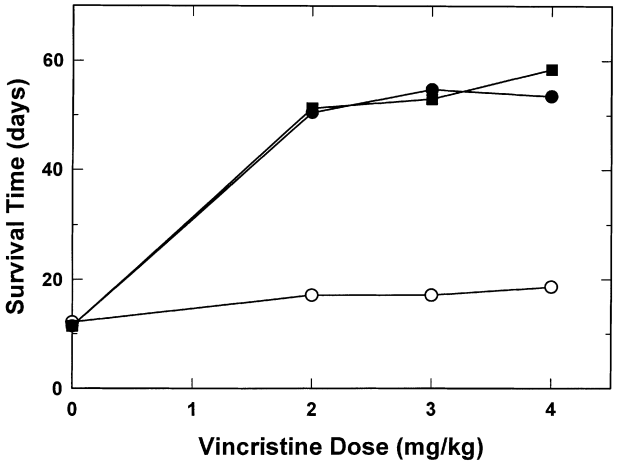

Fig. 3 Dose dependence of the P388 antitumor activity of unencapsulated vincristine and liposomal vincristine. BDF1 mice bearing P388 ascitic tumors were treated with a single dose of either unencapsulated vincristine (O) or vincristine encapsulated in SM/chol liposomes at final drug/lipid ratios of either 0.05 (●) or 0.10 (■). Data points are plotted from the data presented in Table 3 from ten mice

Table 4 Summary of the toxicities associated with the administration of unencapsulated vincristine, SM/chol liposomal vincristine (at drug/lipid ratio 0.05), empty SM/chol liposomes and in the no-treatment controls (*n.d.* not determined)

Formulation	Vincristine dose (mg/kg)	Lipid dose (mg/kg)	Frequency of lethality		Frequency of neurotoxicities[a]		% weight change (day 5)	
			Males	Females	Males	Females	Males	Females
Unencapsulated vincristine	2.0	0	0/5	0/5	0/5	0/5	−8.3	−9.5
	4.0	0	5/5	5/5	4/5	3/5	−30.1	−36.8
	6.0	0	5/5	5/5	5/5	3/5	−25.0	−31.5
SM/chol vincristine	2.0	40	0/5	0/5	0/5	0/5	−8.3	0.0
	4.0	80	5/5	4/5	2/5	3/5	−20.8	−27.3
	6.0	120	5/5	5/5	0/5	3/5	n.d.	n.d.
Empty SM/chol	0	120	0/5	0/5	0/5	0/5	+3.6	+5.6
No treatment	0	0	0/5	0/5	0/5	0/5	+3.8	+5.0

[a] Neurotoxicities were defined as the occurrence of splayed legs, poor coordination, paralysis or crawling

Prior to their death, severe neurotoxicity (ataxia, lack of rear limb control, splayed legs etc.) was observed in seven of ten animals at the 4 mg/kg dose and eight of ten animals at the 6 mg/kg dose. In mice receiving 2 mg/kg of liposomal vincristine, there was no evidence of toxicity or lethality immediately after injection, although slight weight loss, and subsequent recovery, was observed in these animals. Physical abnormalities at this dose consisted only of nonspecific manifestations of toxicity (e.g. hunchbacked appearance, roughened hair coat) and were only observed in male mice. At the 4 mg/kg dose of liposomal vincristine, 50% of the mice displayed short-term (30–60 s) side effects immediately after bolus injection and neurologic toxicity was seen in five of the ten mice at this dose (Table 4). The lethality of liposomal vincristine at 4 mg/kg was 90%. At the 6 mg/kg dose of liposomal vincristine, nine of ten animals displayed acute distress similar in nature to that seen after a similar dose of empty liposomes. Manifestations of neurologic toxicities were seen in only three of the ten mice treated at this high liposomal vincristine dose, but the lethality was 100% for this treatment group (Table 4).

In contrast to both the unencapsulated vincristine and liposomal vincristine-treated mice, no acute lethality was observed in untreated animals or in those receiving empty SM/chol liposomes (Table 4). Furthermore, no weight loss and none of the primary manifestations of neurotoxicities (splayed legs, poor coordination, paralysis or crawling) were observed in untreated animals or in animals that had been treated with empty SM/chol liposomes. In animals that received the empty SM/chol liposomes, acute signs of distress (spastic body movements, unsteadiness, convulsions, or momentary loss of consciousness) did occur in eight of ten animals. All signs abated shortly (1–15 min) after intravenous bolus injection and the animals appeared normal thereafter.

In summary, the liposomal vincristine formulations were somewhat less toxic than unencapsulated vincristine. For example, at 4 mg/kg, the frequency of neurologic toxicities was 70% in the group receiving unencapsulated drug vs 50% in the group receiving liposomal vincristine. At 6 mg/kg, the frequency of neurologic toxicities in the unencapsulated vincristine treatment group was 80%, but was only 30% in the group treated with liposomal vincristine. However, there was no difference in the nadir of weight loss or the duration of weight loss between those animals treated with unencapsulated vincristine and those treated with liposomal vincristine.

Discussion

The objective of this study was to identify an optimal liposomal formulation of vincristine for development in phase I/II clinical trials. For vincristine, previous work has demonstrated that encapsulation in liposomes significantly improves the drug circulation lifetime and antitumor activity [4, 9, 10, 12, 14, 15]. While encapsulation in SM/chol liposomes at a drug/lipid ratio of 0.10 improves drug pharmacokinetics and efficacy [15], we anticipated that the optimum drug/lipid ratio for formulation stability would be at the lower value of 0.05 [10] because the higher lipid dose associated with this lower drug/lipid ratio might confer longer circulation lifetimes due to reduced RES clearance. The experimental objectives of this study were to characterize the alterations in vincristine blood clearance, biodistribution, antitumor activity and acute toxicity that occurred as a consequence of encapsulation in SM/chol liposomes at drug/lipid ratios of 0.05 and 0.10.

The encapsulation of vincristine in SM/chol liposomes at both drug/lipid ratios resulted in significant increases in vincristine concentrations and circulation lifetimes in the blood compartment compared to unencapsulated drug (Fig. 1). These increased blood and

plasma concentrations of vincristine were also associated with increased vincristine accumulation in a variety of tissues (Tables 1 and 2) and with significant improvements in activity against the murine P388 ascitic tumor model. For example, at 2 mg/kg vincristine the unencapsulated drug resulted in a 33% increase in lifespan and no long-term survivors while an identical dose of SM/chol liposomal vincristine (at either drug/lipid ratio) resulted in an increase in lifespan of more than 400% and a long-term survival rate in excess of 50% (Table 3).

An interesting feature of the data reported here is the similarity of the circulation lifetimes (Fig. 1), drug disposition (Fig. 2 and Tables 1 and 2) and antitumor activity (Fig. 3 and Table 3) for the formulations at the two different drug/lipid ratios. This result indicates that the accumulation of vincristine in tissues was not limited by a maximal tissue accumulation of liposomes since, if such a maximum existed, the preparation with a 50% lower drug/lipid ratio (i.e. 0.05) would be expected to have a 50% reduction in drug accumulation. This reduction was not observed at these lipid doses (Tables 1 and 2). Similarly, the accumulation of liposomes and liposomal drug at tumor sites occurs by an extravasation process through damaged and leaky vasculature [3]. While it might be anticipated that liposomes with a higher drug/lipid ratio (i.e. 0.10) would carry greater therapeutic benefit per liposome and have greater antitumor activity, this study demonstrated no differences in the efficacy of the two different liposomal formulations (Table 3 and Fig. 3).

The importance of these observations is that these liposomes encapsulating vincristine are displaying dose-independent clearance. That is, the pharmacologic behavior of the 0.05 drug/lipid ratio formulation was identical to that of the 0.10 drug/lipid ratio formulation, in spite of its administration at twice the lipid dose of the latter liposomes. However, it is typically accepted that dose-independent clearance is a characteristic of polyethylene glycol-coated liposomes that display extended circulation lifetimes whereas dose-dependent clearance is characteristic of liposomes lacking polyethylene glycol [1]. The likely explanation for the dose-independent behavior of the liposomal vincristine formulations is that the encapsulated vincristine causes an inhibition of uptake by RES cells (RES blockade) as observed for liposomal doxorubicin [2].

Since the encapsulation of vincristine in liposomes significantly increased the exposure of a wide variety of organs to vincristine (Tables 1 and 2), it might be predicted that encapsulation would also result in a concomitant increase in acute drug toxicity. Instead, no increases in toxicity, as manifested by increased lethality, more extensive and/or prolonged weight loss, or signs of neurologic impairment, were observed in direct comparisons of unencapsulated vincristine and liposomal vincristine (Table 4). The significant increases in total drug exposure of 10- to 30-fold observed in many tissues (Table 2) were small in comparison to the 300-fold increases in total drug exposure in the plasma that were due to encapsulation ($AUC_{liposomal}/AUC_{unencapsulated}$; Table 2). These data strongly suggest that the accumulation of vincristine in tissues was dependent on liposome extravasation from the circulation and accumulation in the organ. However, these data also indicate that the liposomal vincristine in the tissues was not readily bioavailable, or was bioavailable with kinetics insufficient to provoke an acute toxicity response. This conclusion is consistent with the observation that the vincristine present in the blood compartment was completely accounted for by the drug retained within the liposomes.

Taken in sum, these data demonstrate that the encapsulation of vincristine in SM/chol liposomes, at either drug/lipid ratio, dramatically increased the therapeutic index for vincristine activity against the murine P388 ascitic tumor model. Encapsulation of vincristine conferred a dramatic increase in drug concentrations in the blood compartment and this pharmacokinetic parameter was strongly correlated with the extent of improvement in antitumor activity. Encapsulation was also associated with increased drug exposure of a wide variety of organs, but this exposure was not correlated with increased drug-associated toxicities. For this SM/chol liposomal anticancer drug, the choice of an optimal formulation for development in clinical trials may be based to a greater extent on process development, manufacturing and shelf-life stability considerations, than on pharmacokinetic, toxicologic or anticancer efficacy considerations.

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References

1. Allen TM, Hansen C (1991) Pharmacokinetics of stealth versus conventional liposomes: effect of dose. Biochim Biophys Acta 1068: 133
2. Bally MB, Nayar R, Masin D, Hope MJ, Cullis PR, Mayer LD (1990) Liposomes with entrapped doxorubicin exhibit extended blood residence times. Biochim Biophys Acta 1023: 133
3. Bally MB, Masin D, Nayar R, Cullis PR, Mayer LD (1994) Transfer of liposomal drug carriers from the blood to the peritoneal cavity of normal and ascitic tumor-bearing mice. Cancer Chemother Pharmacol 34: 137
4. Boman NL, Masin D, Mayer LD, Cullis PR, Bally MB (1994) Liposomal vincristine which exhibits increased drug retention and increased circulation longevity cures mice bearing P388 tumors. Cancer Res 54: 2830
5. Gorski J, Nowak J, Bautembach S, Czestochowska E, Tyminski W (1990) Evaluation of the stability of vincristine binding to blood platelets by the method of spin labeling of platelet membranes. Acta Haematol Pol 21: 46
6. Kanter PM, Klaich GM, Bullard GA, King JM, Bally MB, Mayer LD (1994) Liposome encapsulated vincristine: preclinical toxicologic and pharmacologic comparison with free vincristine and empty liposomes in mice, rats and dogs. Anticancer Drugs 5: 579
7. Mayer LD, St.-Onge G (1995) Determination of free and liposome-associated doxorubicin and vincristine levels in

plasma under equilibrium conditions employing ultrafiltration techniques. Anal Biochem 232: 149
8. Mayer LD, Tai LCL, Ko DSC, Masin D, Ginsberg RS, Cullis PR, Bally MB (1989) Influence of vesicle size, lipid composition, and drug-to-lipid ratio on the biological activity of liposomal doxorubicin in mice. Cancer Res 49: 5922
9. Mayer LD, Bally MB, Loughrey H, Masin D, Cullis PR (1990) Liposomal vincristine preparations which exhibit decreased drug toxicity and increased activity against murine L1210 and P388 tumors. Cancer Res 50: 575
10. Mayer LD, Nayar R, Thies RL, Boman NL, Cullis PR, Bally MB (1993) Identification of vesicle properties that enhance the antitumour activity of liposomal vincristine against murine L1210 leukemia. Cancer Chemother Pharmacol 33: 17
11. Mayer LD, Gelmon K, Cullis PR, Boman N, Webb MS, Embree L, Tolcher T, Bally MB (1995) Preclinical and clinical studies with liposomal vincristine. Progress in drug delivery systems IV. Biomedical Research Foundation, Tokyo, p 151
12. Mayer LD, Masin D, Nayar R, Boman NL, Bally MB (1995) Pharmacology of liposomal vincristine in mice bearing L1210 ascitic and B16/BL6 solid tumors. Br J Cancer 71: 482
13. Sethi VS, Jackson DV Jr, White DR, Richards F 2d, Stuart JJ, Muss HB, Cooper MR, Spurr CL (1981) Pharmacokinetics of vincristine sulphate in adult cancer patients. Cancer Res 41: 3551
14. Vaage J, Donovan D, Mayhew E, Uster P, Woodle M (1993) Therapy of mouse mammary carcinomas with vincristine and doxorubicin encapsulated in sterically stabilized liposomes. Int J Cancer 54: 959
15. Webb MS, Harasym TO, Masin D, Bally MB, Mayer LD (1995) Sphingomyelin-cholesterol liposomes significantly enhance the pharmacokinetic and therapeutic properties of vincristine in murine and human tumour models. Br J Cancer 72: 896