International Journal of Pharmaceutics 391 (2010) 230–236

Contents lists available at ScienceDirect

International Journal of Pharmaceutics

journal homepage: www.elsevier.com/locate/ijpharm

Development of pegylated liposomal vinorelbine formulation using "post-insertion" technology

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ARTICLE INFO

Article history:
Received 11 January 2010
Accepted 2 March 2010
Available online 7 March 2010

Keywords:
Liposomes
Vinorelbine
DSPE-PEG
Post-insertion
Active loading
Pharmacokinetics
Toxicity
Efficacy

ABSTRACT

Prolonged vinorelbine exposure is correlated with improved antineoplastic effects, as evidenced by increased response rate in patients receiving continuous infusion. The administration of slow release pegylated liposomal vinorelbine formulation might mimic the pharmacokinetics of a continuous infusion, thus improving antitumor efficacy. But it is hard to prepare pegylated liposome vinorelbine using DSPE-PEG (an extensively used peglipid) because it could induce accelerated drug release. To resolve this problem, "post-insertion" technology was employed to prepare pegylated liposome vinorelbine formulations, which involved the incubation of vinorelbine-containing vesicles with DSPE-PEG micellar solutions. HPLC analysis revealed that after incubation at 60 °C for 60 min, ~100% DSPE-PEG could be inserted into the outer monolayer of the vesicles. Moreover, the grafting of peglipid did not induce the release of entrapped vinorelbine irrespective of intraliposomal anions. Drug release experiments indicated that "post-insertion" formulations were more able to retain entrapped drugs than "co-dissolving" formulations. The same phenomenon was observed when both series of formulations were injected in normal mice to compare pharmacokinetic profiles. In L1210 ascitic model, a "post-insertion" formulation with a PEG grafting density of ~0.5% exhibited the strongest antineoplastic effects, thus it was chosen to be further evaluated in S-180 and RM-1 models, in which the formulation was still more therapeutically active than conventional formulations. In conclusion, using "post-insertion" technology, the potential interaction between DSPE-PEG and vinorelbine could be prevented, thus making it possible to develop pegylated vinorelbine formulations.

1. Introduction

Vinorelbine is a semisynthetic vinca alkaloid that is indicated as a single agent or in combination with cisplatin for the first line treatment of patients with advanced non-small cell lung cancer (NSCLC) (Jones and Burris, 1996). The vinca alkaloids are structurally similar compounds comprised of 2 multiringed units, vindoline and catharanthine. Unlike other vinca alkaloids, the catharanthine unit is the site of structural modification for vinorelbine. It has been revealed that the chemical modification resulted in altered chemophysical properties (e.g., increased lipophilicity and membrane permeability), changed toxicity and efficacy profiles relative to other vinca alkaloids (Crawford, 1996; Johnson, 1996; Johnson et al., 1996; Krikorian and Breillout, 1991; Sorensen, 1992).

All the vinca alkaloids are spindle poisons, which interfere with the polymerization of tubulin, the protein responsible for building the microtubule system that is essential to nerve conduction and also the mitotic spindle, which appears during cell division. In terms of cytotoxic action, this results in accumulation of cells at G2/M in the cell cycle (Crawford, 1996; Johnson, 1996; Johnson et al., 1996; Krikorian and Breillout, 1991; Sorensen, 1992). Due to its mechanism of action and cell cycle specificity, prolonged vinorelbine exposure is critical to achieve the optimum therapeutic effects (Aapro et al., 2001).

To realize this purpose, the employment of liposomal drug delivery system might be an option. The slow release of drugs from vesicles could guarantee the prolonged exposure of tumor cells to the released drugs. Moreover, the selective biodistribution of vinorelbine after liposome encapsulation might reduce the toxicity of vinorelbine (Chow et al., 2008, 2009; Drummond et al., 2009; Nabiev et al., 1998; Semple et al., 2005; Webb et al., 2007;

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doi:10.1016/j.ijpharm.2010.03.004

Zhigaltsev et al., 2005, 2006). Although vinorelbine possesses relative selectivity for mitotic microtubules in comparison with other vinca alkaloids, it still exhibits other side effects in clinical practice (e.g., severe granulocytopenia) (Curran and Plosker, 2002; Gregory and Smith, 2000; Karminsky et al., 1999).

However, it is not easy to achieve liposomal vinorelbine formulation with improved therapeutic index without the development of novel strategies. Due to chemical modification, vinorelbine becomes more lipophilic and membrane permeable (Zhigaltsev et al., 2005, 2006), thus posing a challenge for effective retention of vinorelbine inside vesicles. In addition, to improve drug targeting and long-term stability, the modification of vesicles with peglipids might be indispensable, but it is reported that DSPE-PEG (an extensively used peglipid) might induce the accelerated release of vincristine from liposomes (Webb et al., 1998). Since vinorelbine is structurally similar to vincristine, the same phenomenon might occur. Therefore, to develop pegylated liposome formulation, these two obstacles must be resolved first.

In previous study, we have found that certain kinds of anions (such as 5-sulfosalicylate) could form stable aggregates with vinorelbine, thus improving vinorelbine retention. Despite that the result was encouraging, novel strategy that permits modification of vesicles with DSPE-PEG is still desirable. In this study, we mainly investigated the influences of anions, PEG grafting density and pegylation method on vinorelbine loading, release and retention. It is found that the employment of 5-ssa as trapping agents, and modification of vesicles via "post-insertion" technology could markedly improve the retention of vinorelbine. Accordingly, a pegylated liposomal vinorelbine formulation was developed, which exhibited significantly improved antitumor efficacy in L1210, S-180 and RM-1 tumor models.

2. Materials and methods

2.1. Materials

Vinorelbine bitartrate was provided by Hainan Jiamao Plant Development Co., Ltd. (Hainan, China). Hydrogenated soybean phosphatidylcholine (HSPC) was a kind gift from Degussa (Freising, Germany). N-(Carbonyl-methoxypolyethyleneglycol$_{2000}$)-1,2-distearoyl-sn-glycero-3-phosphoethanolamine, sodium salt (DSPE-PEG) was obtained from Genzyme Pharmaceuticals (Liestal, Switzerland). Cholesterol (Chol), Sepharose 4B and Sephadex G-75 (medium) were obtained from the Sigma Chemical Company (St. Louis, MO). Nucleopore polycarbonate filters (47 mm, 0.1 μm pore sizes) were obtained from Northernlipids, Inc. (Canada). All other chemicals used in this study were analytical or high-performance liquid chromatography (HPLC) grade.

The S-180, L1210 and RM-1 tumor cell lines were originally purchased from Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences (Shanghai, China). KM mice (8–10 weeks old) were obtained from Hebei Medical University. BDF1 and c57 mice were purchased from Vitalriver (Beijing, China).

2.2. Preparation of liposomes

Liposomes were prepared according to the following procedure. Briefly, the mixtures of HSPC, Chol and DSPE-PEG were solubilized in chloroform and dried to a thin lipid film under a stream of N_2 gas, followed by incubation overnight under vacuum to remove residual solvent. In all cases, the molar ratio of HSPC to Chol was set as 3:2, but the DSPE-PEG/HSPC molar ratio might be 0, 0.5, 2.9 and 8.3%. The dried lipid films were subsequently hydrated with 300 mM desired ammonium salt solutions (e.g., sulfate, *p*-phenolsulfonate, 5-sulfosalicylate, phosphate, phytate). The hydration process was performed at 60 °C for 1 h. The dispersion was extruded eight times through polycarbonate filters of 0.10 μm employing a LiposoFast-100 jacketed extruder obtained from Avestin (Ottawa, Canada) at 60 °C. This procedure formed unilamellar vesicles of ~100 nm.

The zeta average size of vesicles was analyzed using quasi-elastic light scattering (Zetasizer Nano-ZS; Malvern Instruments, UK). Before analysis, the samples were diluted in 0.9% NaCl with a volume ratio of 1/200. The zeta potential of vesicles was also determined using Nano-ZS, but the measurement was carried out in water after 30-fold dilution. In both cases, DTS4.0 software was used to collect the data that were analyzed using "multinarrow modes".

2.3. Remote loading of liposomes

A transmembrane ammonium salt gradient was generated across the vesicles by exchanging the extraliposomal buffer using Sephadex G-75 columns. The buffer employed in the experiments was sucrose (300 mM)–histidine (20 mM) buffer (pH 7.5). Upon buffer exchange, empty liposomes with transmembrane ammonium salt gradient were mixed with concentrated vinorelbine solutions (10:1, v/v), resulting in a desired mass ratio. The resulting mixture was incubated at 60 °C for 40 min to realize drug loading. After loading, the liposomal preparations were concentrated to a vinorelbine concentration of 2 mg/mL using a Millipore Labscale TFF System (with 50,000 nominal molecular weight limit polysulfone filters).

For determining the loading efficiency, samples of the mixtures were taken and unentrapped vinorelbine was removed by size exclusion chromatography. Briefly, 100 μL samples were loaded onto Sephadex G-75 mini-column (56 mm × 8 mm i.d.), and then eluted using 0.9% NaCl solution.

2.4. Modification of vesicles with peglipids via "post-insertion" (Allen et al., 1991; Iden and Allen, 2001; Moreira et al., 2002)

Prior to experiments, liposomes and a concentrated micellar DSPE-PEG were first equilibrating to 60 °C. The insertion of peglipids into vesicles was started by mixing aliquots to give a final theoretical 0.5, 2.9 and 8.3 mol% of DSPE-PEG to HSPC in the outer monolayer. After incubation of the mixtures at 60 °C for 60 min, the micelles were separated from the liposomes by Sepharose 4B column chromatography. Liposome fraction in the void volume was collected for lipid analysis.

2.5. Determination of lipid compositions

A Waters HPLC system was used to determine lipid contents (HSPC, peglipid and cholesterol). The system was composed of 2690 liquid chromatograph and 410 RI detector, and controlled by Millennium 32 software. For the measurement of lipid content, a zobax c18 (25 cm × 4.6 mm i.d., 5 μm particle size) was employed, which was maintained at 35 °C during the analysis procedure. The mobile phase was a mixture of methanol, tetrahydrofuran (THF) and 0.17 mol/L ammonium acetate (94:5:1), running at a flow rate of 1 mL/min. The retention times for different components were 4.8 min (DSPE-PEG), 8.6 min (cholesterol), 10.1 min for the first peak of HSPC (PSPC) and 15.3 min for the second peak of HSPC (DSPC).

Two milliliters of samples were diluted to 10 mL using chloroform–methanol (1:2) mixture, and then the resulting solutions were injected into HPLC with injection volume of 10 μL for the analysis of lipid compositions.

2.6. Liposomal formulations

A total of seven liposomal vinorelbine formulations were prepared, which were modified at different PEG grafting density using different peglipid insertion technology. For liposomes prepared with "post-insertion" technology, the molar percent of peglipids to HSPC were 0.25, 1.45 and 4.15, respectively. In contrast, the formulations prepared by "co-dissolving peglipid with other lipids" contained 0.5, 2.9 and 8.3 mol% DSPE-PEG, respectively (Kenworthy et al., 1995; Needham et al., 1997). Since only the outer monolayer was modified in "post-insertion" formulation and in "co-dissolving" formulation both sides of membrane were grafted with peglipids, these two series of formulations should have the same PEG grafting density in the outer monolayer. For all the formulations, the drug to total lipid mass ratio was 1/8 and ~100% vinorelbine was loaded into vesicles.

The resultant formulations were named lv-pi0.5, lv-pi2.9, lv-pi8.3, lv-cd0.5, lv-cd2.9, lv-cd8.3 and lv-c. Here lv meant liposomal vinorelbine; pi and cd were the abbreviations of "post-insertion" and "co-dissolving". The numbers referred to mol% PEG grafting density. Formulation lv-c was conventional liposomes without PEG modification.

2.7. In vitro release studies (Cui et al., 2007; Nabiev et al., 1998)

Liposomal vinorelbine formulations, at a concentration of 0.46 mM HSPC, were diluted in release buffer (glucose/histidine/NH_4Cl, 300/10/5 mM). Then 2 mL of diluted liposomes were placed in the dialysis tubing with a molecular weight cutoff of 10 kDa, and dialyzed against 400 mL of release buffer containing penicillin and streptomycin (100 μg/mL for each antibiotics). The dialysis process was performed at 37 °C. At various time points, aliquots were withdrawn and stored at −20 °C until analysis. The samples were treated and analyzed using the method mentioned in PK studies.

2.8. Pharmacokinetic studies

Plasma pharmacokinetic analysis was performed in normal KM mice. For PK studies, KM mice received injections of 10 mg/kg single i.v. bolus dose of liposomal vinorelbine formulations via tail vein. At the indicated time points, blood samples were obtained via cardiac puncture under anesthesia and collected in Eppendorf tubes containing sodium heparin as an anticoagulant. Blood samples were centrifuged at 2500 rpm for 10 min to separate the plasma. The plasma samples were stored at −20 °C until additional analysis.

Vinorelbine concentrations in plasma samples were determined using HPLC method. For 20 μL Plasma, 20 μL purified water and 460 μL methanol was added. The resulting mixture was vortexed and permitted to precipitate at −20 °C for at least 1 h; and then centrifuged at 20,000 × *g* for 10 min. The supernatant was collected for analysis. The injection volume for samples was 20 μL.

A Waters HPLC system controlled by Millennium 32 software was used for chromatographic analysis, which was composed of 2690 liquid chromatograph and 996 diode array detector. The HPLC separations were achieved using a Zorbax C18, 150 mm × 4 mm i.d., 5 μm particle size column. The isocratic mobile phase was a mixture of methanol and 80 mM ammonium acetate (55/45, v/v; the pH of aqueous phase was adjusted with HCl to 3.0), running at a flow rate of 1 mL/min. Detection was accomplished at 264 nm. The retention time for vinorelbine was ~6 min, the recovery of drug was >95% and the standard curve with an *r* value of 0.999. The pharmacokinetic variables were calculated using DAS 2.0 software (the net for drug evaluation of China).

2.9. Antitumor efficacy studies

Male BDF1 mice were inoculated i.p. with 5×10^5 L1210 murine tumor cells, derived from the ascitic fluid of a previously infected BDF1 mouse. Free vinorelbine or liposome-encapsulated vinorelbine were administrated via a lateral tail vein, 24 h after tumor cell inoculation. Animal weights were monitored daily and mortality was determined up to 60 days. Because death cannot be used as an end point, mice were sacrificed at the first sign of distress. The data was analyzed with SPSS 11.5 version software (survival analysis).

RM-1 prostate (or S-180 sarcoma) tumor cells were injected s.c. (5×10^5 cells/mouse) in the right flank region of c57 (or KM) mice. Tumors were allowed to grow to a mean tumor volume of 0.2 cm^3 (for RM-1 model) or 0.4 cm^3 (for S-180 model) before initiation of treatment. Tumor-bearing mice were randomly divided into different groups ($n = 11$ or 10). The treatment groups were given a single i.v. injection of liposomal vinorelbines (10 mg/kg), respectively. Control mice were treated with an isotonic sucrose–histidine solution. The tumor volume (V) was calculated according to the equation $(\pi/6) \times \text{width}^2 \times \text{length}$. Animal weight and tumor sizes were monitored every 2 (or 3) days. For statistical analysis, the tumor volumes of treatment group and control at each time point were examined using one-way analysis of variance. Post hoc comparison of the means of individual groups was performed using LSD test. In all cases, $p < 0.05$ was considered to be statistically significant.

3. Results

3.1. Preparation and characterization of liposomes

All the empty liposomes were prepared by hydration of dried lipid membrane followed by extrusion through 0.1 μm pores. Size measurement revealed that the resultant vesicles had a narrow size distribution (polydispersion index <0.05) and mean vesicle size ranged from 100.3 to 105.8 nm, similar to the pore size of the membrane. The influences of drug loading and pegylation on vesicle size and size distribution were negligible, despite that it was reported that vesicle size slightly increased with increasing PEG grafting density.

In contrast, PEG grafting density markedly affected the zeta potentials of vesicles. The vesicles became more negatively charged as the mol% PEG increased irrespective of whether the vesicles were prepared using co-dissolving method or not. For example, when the PEG density elevated from 0.5 to 8.3%, the zeta potential increased correspondingly from -26.0 ± 0.1 to -39.0 ± 0.6 mV.

The zeta potentials of "pi" vesicles were similar to those of "cd" vesicles when the outer monolayer was grafted with the same amount of peglipids. The analysis of peglipid content using HPLC method revealed that peglipids could be completely inserted into lipid bilayers after co-incubation of liposomes with peglipid micellar solutions at 60 °C for 60 min.

3.2. The effects of peglipid on vinorelbine loading

Two series of liposome formulations were prepared, one was grafted with 8.3% peglipid on both surfaces of the vesicles, and the other contained no peglipids. Ammonium salts of different anions (sulfate, *p*-phenolsulfonate, 5-sulfosalicylate, phosphate and phytate) were entrapped into vesicles to create a transmembrane ammonium gradient.

Vinorelbine was added into empty liposomes exhibiting a transmembrane gradient in a D/L ratio of 2/9.58 (mg/mg), and incubated at 60 °C for different time to achieve drug loading profiles. By comparing drug loading profiles, it is easy to find that the modification of lipid surfaces with DSPE-PEG could affect drug loading

Table 1
Effects of anions and grafted PEG on vinorelbine loading. Both conventional liposomes and pegylated liposomes (co-dissolving method) were prepared by hydration of dried lipid membranes (HSPC/Chol, HSPC/Chol/DSPE-PEG) with ammonium salt solutions followed by extrusion of 100 nm pores. To create transmembrane gradient, the external buffers were exchanged with SHB (300 mM sucrose and 20 mM histidine, pH 7.5). For drug loading, liposomes were incubated with vinorelbine with a drug to HSPC mass ratio of 0.2 at 60 °C for 40 min. And then conventional liposomes with entrapped vinorelbine were incubated with peglipid micellar solution at t 60 °C for 60 min, resulting in the modification of outer monolayer with 8.3 mol% peglipids ("post-insertion" method). At specified time points, the EE% was determined as described in Section 2. In all cases, the RSD values were (<2%) and only mean values were presented.

Anions	% loading of vinorelbine		
	Vesicles without DSPE-PEG	Pegylated vesicles with 8.3 mol% DSPE-PEG	
		Co-dissolving method	Post-insertion method
Sulfate	98.4	91.6	99.6
p-Phenolsulfonate	99.7	5.4	100
5-Sulfosalicylate	100	100	100
Phosphate	89.0	50.1	88.7
Phytate	99.4	80.4	99.1

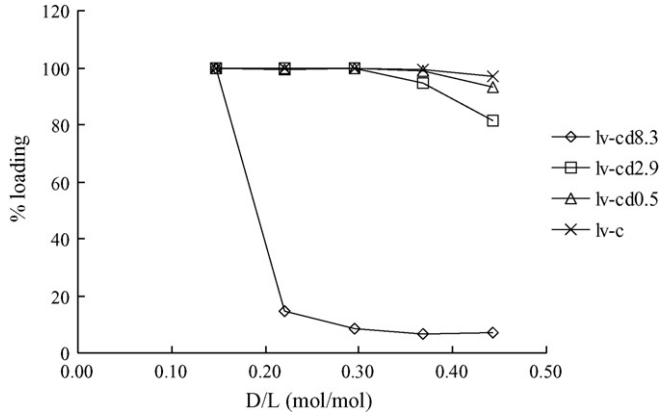

Fig. 1. Effects of drug to lipid ratio and grafted PEG on vinorelbine loading. Both conventional liposomes and pegylated liposomes (co-dissolving method, mol% peglipid: 0.5, 2.9 and 8.3) were prepared by hydration of dried lipid membranes (HSPC/Chol, HSPC/Chol/DSPE-PEG) with ammonium 5-sulfosalicylate solution followed by extrusion of 100 nm pores. To create transmembrane gradient, the external buffers were exchanged with SHB (300 mM sucrose and 20 mM histidine, pH 7.5). For drug loading, liposomes were incubated with vinorelbine with varied drug to lipid ratio (mol/mol) at 60 °C for 40 min. At specified time points, the EE% was determined as described in Section 2. In all cases, the RSD values are (<2%) and only mean values are presented in the figure.

to a varying degree (Table 1). In the presence of peglipids, ammonium phenolsulfate mediated vinorelbine loading was unstable, after 60 min incubation, >90% entrapped vinorelbine was lost from vesicles. Encouragingly, the grafted PEG has no influences on vinorelbine loading into 5-sulfosalicylate vesicles. For other vesicles, the decrease of % loading increased as the internal buffer changed as the following: ammonium phosphate (~40%) > phytic acid (~20%) > $(NH_4)_2SO_4$ (~10%). But in these cases, the loading was stable, despite the loading efficacy decreased.

Further experiments proved that when D/L mass ratio increased, the loading of vinorelbine into 5-ssa vesicles with high PEG content (both sides) was also affected. Fig. 1 presents the drug loading profiles into vesicles encapsulating 5-ssa at different D/L ratio and PEG molar percent. It clearly showed that when D/L ratio was >3 and the molar percent of peglipids was ~8.3%, the loading efficiency significantly decreased to ~15%. These results revealed that even when 5-ssa was used as entrapping agent, the direct contact of peglipid with vinorelbine was still detrimental to drug loading.

Surprisingly, the modification of vesicles using "post-insertion" technology did not induce the decrease of encapsulation efficiency. After the grafting of peglipid into the outer monolayer, the % vinorelbine loading remained almost constant. Furthermore, after 4-week storage at 4 °C, the leakage of vinorelbine from "post-insertion" formulations were negligible, irrespective of internal buffer compositions, considerably compared to "co-dissolving" formulations (Table 2). For "cd" formulations, if the anions could not form stable complexes with vinorelbine, significant release of vinorelbine could occur (e.g., phytate).

3.3. In vitro release

Since in nature vinorelbine was loaded into vesicles in response to a transmembrane NH_3 gradient, the addition of free NH_3 in release media could reverse the active gradient, thus intriguing vinorelbine release. Drug release experiments were performed in isotonic, physiological pH buffer containing 5 mM NH_4Cl at 37 °C. The results indicated that for both series of formulations, increased PEG grafting density induced accelerated release from vesicles (Table 3). However, in contrast to "cd" formulations, the "pi" formulations were more able to retain entrapped vinorelbine ($p < 0.05$). Because both series of formulations had the same vesicle size, internal buffer composition and drug to lipid mass ratio, the difference in drug release should be ascribed to PEG grafting density and pegylation method. It seems that the prevention of direct interaction of vinorelbine and peglipid was advantageous.

3.4. Plasma pharmacokinetic studies

As shown in Fig. 2, all the pegylated liposome formulations displayed desired prolonged circulation time compared to conventional liposomes (two-way ANOVA, $p < 0.001$). For "pi" formulations, increased PEG grafting density induced increased

Table 2
Effects of anions and grafted PEG on vinorelbine retention. The liposome formulations were prepared as described in Table 1. After 4 weeks storage at 4 °C, the entrapment efficiency was determined with size exclusion chromatography. In all cases, the RSD values were (<2%) and only mean values were presented.

Anions	% loading of vinorelbine		
	Vesicles without DSPE-PEG	Pegylated vesicles with 8.3 mol% DSPE-PEG	
		Co-dissolving method	Post-insertion method
Sulfate	98.3	91.8	98.6
5-Sulfosalicylate	99.6	99.4	100
Phytate	99.2	36.5	98.6

Table 3
In vitro release studies of liposomal vinorelbine formulations. The liposomes were diluted using the release media (isotonic glucose/histidine/NH_4Cl buffer: 275/10/5 mmol/L, pH 7.5), to a PC concentration of 0.46 μmol/mL; and then 2 mL diluted liposomes were put into dialysis tubing with a molecular weight cutoff of 10,000, and dialyzed against 400 mL release buffers at 37 °C. The samples were withdrawn at specific time points and analyzed using HPLC method described in methods and materials. Each experiment was repeated for at least twice, and the mean values were used to calculate the release parameters. The regression was performed using SPSS software (11.5 version) and the data were fitted into exponential equation was $y = y_0 \times e^{-kt}$, where y_0 is initial encapsulation efficiency (%), y is %EE at time t, and k is for release constant. Half-life was determined by LN(2)/k.

Formulations	r^2 Value	p Value	k	Half-life (h)	b_0
lv-cd0.5	0.931	0.000	.0690	10.05	97.7
lv-cd2.9	0.962	0.000	.0849	8.16	93.5
lv-cd8.3	0.897	0.000	.0904	7.67	99.0
lv-pi0.5	0.936	0.000	.0445	15.58	98.5
lv-pi2.9	0.938	0.000	.0482	14.38	97.2
lv-pi8.3	0.948	0.000	.0761	9.11	98.3

plasma half-times, and two-way ANOVA revealed that PK profiles of this series of formulations were different ($p < 0.01$). The $t_{1/2}$ values for this series of formulations were 12.44, 17.68 and 19.80 h, respectively. On the contrary, the circulation times of "cd" formulations did not prolong as the mol% peglipids increased. This might be related to the accelerated release of vinorelbine when inner and outer monolayers were simultaneously modified with peglipids. Comparing the PK profiles of these two series of formulations, it

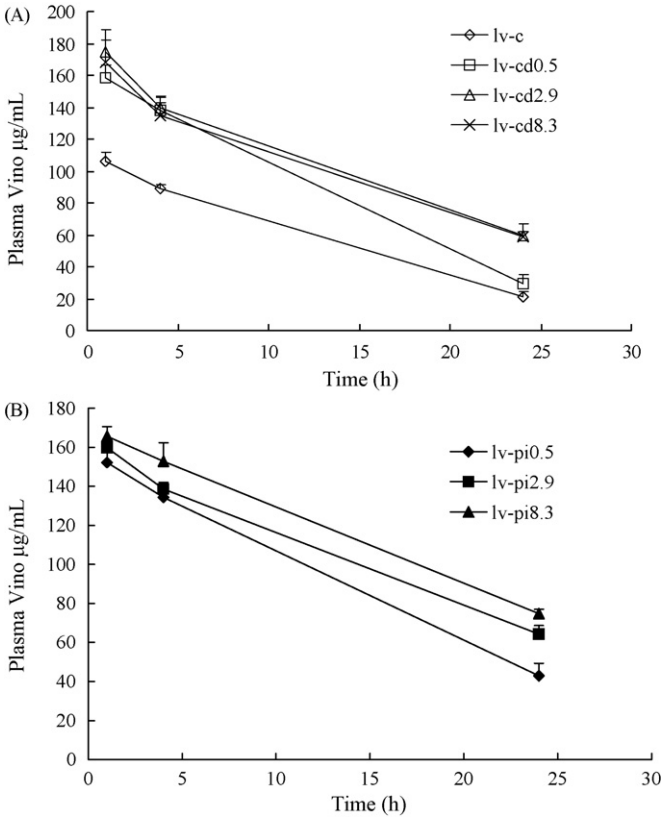

Fig. 2. Plasma pharmacokinetics of liposome vinorelbines in normal KM mice. Liposomal vinorelbine formulations were administrated to KM mice at 10 mg/kg via tail vein ($n = 3$). At specified time points, the mice were sacrificed and the blood was sampled via cardiac puncture. The total plasma vinorelbine concentrations were determined using HPLC method described in methods and materials. Data points represent the mean values calculated from 3 samples. To increase the clarity of the figure, the PK curves following the administration of "co-dissolving" and "post-insertion" formulations are depicted in (A and B), respectively.

Table 4
Pharmacokinetic parameters of different vinorelbine formulations in KM mice. Different liposomal vinorelbine formulations were injected into male KM mice (18–22 g, 6–8 weeks) via lateral tail veins at a dose level of 10 mg/kg (injection volume: 10 mL/kg). At specified time points, the mice were sacrificed and the blood was sampled via cardiac puncture. The total plasma vinorelbine concentrations were determined using HPLC method described in methods and materials. The mean plasma vinorelbine concentration values were used to calculate the pharmacokinetic parameters. To determine the parameters, DAS2.0 software was employed.

Formulations	r^2 Value	p Value	k	Half-life (h)	b_0
lv-pi-0.5	0.999	0.020	0.0557	12.44	164.2
lv-pi-2.9	0.999	0.016	0.0392	17.68	164.3
lv-pi-8.3	0.999	0.018	0.0350	19.80	173.5
lv-cd-0.5	0.998	0.030	0.0750	9.24	177.5
lv-cd-2.9	0.994	0.049	0.0455	15.23	175.8
lv-cd-8.3	0.994	0.051	0.0440	15.75	168.4
lv-c	0.999	0.014	0.0700	9.90	115.6

is easy to find that "post-insertion" technology could improve the retention of vinorelbine relative to "cd" formulations, especially at the highest outer layer PEG grafting density ($p = 0.004$, Table 4).

3.5. Antineoplastic effects in L1210 model

Despite that vinorelbine is not indicated for the treatment of leukemia, murine L1210 leukemia has been shown to response to vinorelbine, thus in our studies, L1210 i.p. model was employed to evaluate the antineoplastic effects of different liposomal formulations. 5×10^5 L1210 cells were inoculated into BDF1 mice via intraperitoneal route and 24 h after inoculation the treatments were initiated. For the animals that were left untreated, the i.p. inoculation of L1210 cells induced the early death of animals with a mean survival time of 8.78 ± 0.32 days. The treatment of animals with 8 mg/kg vinorelbine induced slight increase in both mean and median survival times. But the difference between free vinorelbine and control was not significant. All the treatments with liposomal vinorelbine were effective relative to control group except "pi" formulation with PEG grafting density of 8.3 mol%.

Interestingly, it is found that for "pi" formulations, the antineoplastic effects increased with decreasing PEG grafting density ($p < 0.05$), which implied that vinorelbine release rate still increased as the mol% peglipid increased for "pi" formulations, despite that we cannot directly detect this trend in circulation. When the mol% of peglipids increased to 8.3%, the survival time in liposome group was similar to those of free vinorelbine group, exhibiting no advantages in the treatment of leukemia. In contrast, "pi" formulation with 0.5% peglipid was the most therapeutically active among these liposome formulations with a mean and median survival times of 16.7 and 11.0 days, respectively. Both formulations with 2.9% peglipid exhibited almost equivalent activities and were inferior to conventional non-pegylated formulation despite that the difference was not significant.

Based on above results, free vinorelbine was not as effective as other chemotherapeutic agents (e.g., mitoxantrone) in the treatment of L1210 leukemia. Moreover, the formulations with ~0.5% peglipid might be more promising (Table 5).

3.6. Antineoplastic effects in S-180 tumor model

S-180 was a rapid growth tumor model; 4 days post-inoculation, the tumor volumes were rapidly increased to a mean volume of ~450 mm^3 and then the treatments were initiated. Free vinorelbine had no therapeutic effects since two ANOVA revealed no statistical differences between this group and control group. In contrast, both lv-pi0.5 and lv-c could considerably inhibit the growth of tumors ($p < 0.01$, versus control). The tumor growth curves in both liposome groups were almost superimposed, thus hard to discriminate

Table 5
Antitumor efficacies of vinorelbine formulations against L1210 leukemia cell line in BDF1 mice. Mice were inoculated with 5×10^5 cells i.p. on day 0 and treated on day 1.

Treatment group	Dose (mg/kg)	No. of survivors (day 60)	Mean Survival	Median survival Time[b]	% ILS[a]	L/F[a]
Control	0	0/9	8.78 ± 0.32	8		
Free VINO	8	0/9	9.44 ± 0.29	9.00 ± 0.37	12.5	
lv-c	8	0/10	11.7 ± 0.58	11.0 ± 0.63	37.5	1.22
lv-cd2.9	8	0/10	10.4 ± 0.45	10.0 ± 0.29	25.0	1.11
lv-pi0.5	8	1/10	16.7 ± 4.63	11.0 ± 0.24	37.5	1.22
lv-pi2.9	8	0/10	10.8 ± 0.71	10.0 ± 0.24	25.0	1.11
lv-pi8.3	8	0/10	9.50 ± 0.22	9	12.5	1.00

[a] Values for ILS (increased life span) and liposomal/free (L/F) were calculated using median survival data.
[b] To calculate mean and median survival time, survivors after 60 days were assigned survival times of 60 days.

which formulation was more efficacious. Since the tumor growth was not governed by exponential equation, the doubling time was not calculated (Fig. 3).

3.7. Antineoplastic effects in RM-1 tumor model

The growth rate of RM-1 tumor in c57 mice was slow compared to other allograft tumor model (e.g., S-180 in KM mice). The tumor reached to a volume of ~200 mm^3 2 weeks after inoculation, and then free vinorelbine and liposomal vinorelbine were administrated to animals at a dose of 10 mg vinorelbine/kg. Regression analysis revealed that in all animals (control or treated), the tumor growth was governed by exponential equation ($V_t = V_0 e^{kt}$, wherein, V is tumor volume, t is time and k is growth constant.). Both lv-pi0.5 and lv-c could significantly inhibit the tumor growth (Fig. 4). One-way ANOVA revealed that since 5 days post-treatment the tumor volumes in these two groups were markedly smaller than those of the control and this trend maintained till the termination of experiment ($p < 0.001$). However, at the same dose lv-pi0.5 was more therapeutically active than lv-c despite that no statistical difference was found ($p > 0.05$). The tumor doubling time in animals treated with lv-pi0.5 was 5.80 days, about 1.25-fold of that of lv-c group (Table 6).

4. Discussion

"Stealth technology" represents a milestone breakthrough in liposome field since the modification of liposomes with hydrophilic polymers such as PEG could prolong the circulation time and facilitate the passive accumulation of vesicles into tumors (Cattel et al., 2003; Siegal et al., 1995; Takeuchi et al., 2001; Vail et al., 2002).

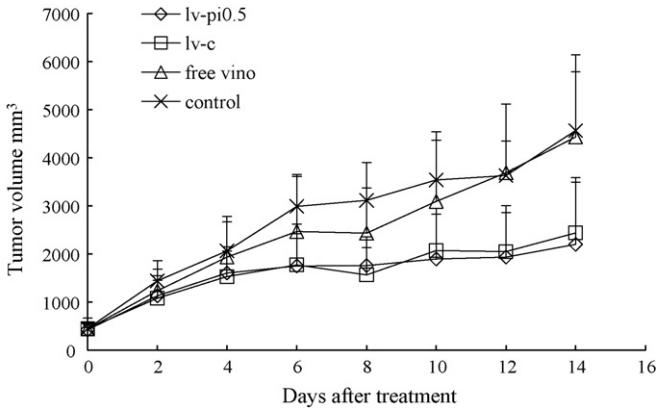

Fig. 3. The antineoplastic effects of liposomal vinorelbine and free vinorelbine in S-180 tumor model. S-180 tumor cancer cell was inoculated into mouse with 5×10^5 cells/mouse. When the tumor volume reached to 0.4 cm^3 (4 days post-inoculation), vinorelbine formulations were injected into KM mice via tail vein at 10 mg/kg. Each data point represents the mean calculated from 10 tumors and the error bar represents standard deviation.

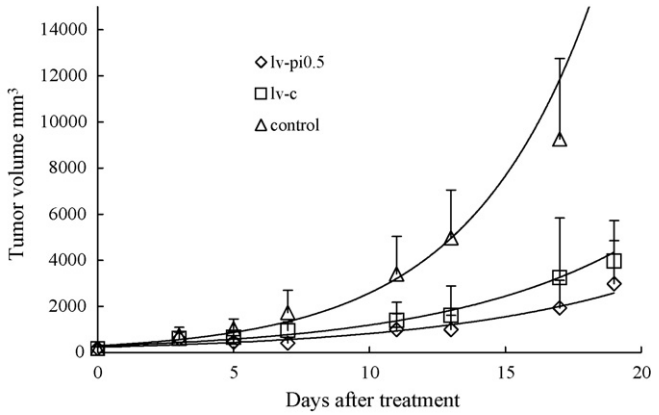

Fig. 4. The antineoplastic effect of liposomal vinorelbine formulations in RM-1 tumor model. When the tumor volume reached to ~0.2 cm^3, vinorelbine formulations were injected into c57 mice via tail vein at 10 mg/kg ($n = 11$). To reveal the exponential growth of tumor, the tumor volume was plotted as a function of time, and the exponential trend lines were added.

Typically, the modification is realized by incorporating the anionic PEG derivative, DSPE-PEG, into lipid membranes. However, it is found that DSPE-PEG had a negative effect on vinorelbine loading, retention and release.

The reason why negatively charged DSPE-PEG increased vinorelbine leakage and affected vinorelbine loading is not clear, but it might be associated with the electrostatic interactions between positively charged vinorelbine and negatively charged DSPE-PEG. To what degree the vinorelbine loading was affected is related to the kinds of entrapped anions. Certain anions (e.g., 5-sulfosalicylate) could mediate effective and stable vinorelbine loading at low D/L ratio and PEG grafting density.

It is not surprising to observe this phenomenon. In our method, vinorelbine was also loaded in response to a pH gradient, which was created in a manner similar to that of ammonium sulfate gradient method (Haran et al., 1993; Lasic et al., 1995). Namely, the transmembrane movement of neutral ammonium leads to the acidification of intraliposomal medium, which promotes the intravesicle accumulation of vinorelbine and its protonation. If anions could form certain aggregation status with protonated vinorelbine,

Table 6
The exponential growth of RM-1 tumor in c57 mice. RM-1 was inoculated into c57 with 5×10^5 cells/mouse. Fourteen days post-inoculation, liposomal vinorelbines were administrated to c57 mice via tail vein at a dose of 10 mg/kg (11 mice/group). The mean tumor volume values were used to calculate the kinetic parameters ($V = V_0 \times e^{kt}$). The doubling time was calculated with LN(2)/k.

Treatment group	Dose level (mg/kg)	r^2	p	V_0	k	Doubling time
Control	0	0.952	0.000	291.7	0.2179	3.18
lv-c	10	0.917	0.001	273.9	0.1490	4.65
lv-pi0.5	10	0.852	0.003	239.7	0.1195	5.80

they could stabilize drug loading. It is possible that 5-ssa could form stable aggregates with vinorelbine compared to other anions, thus preventing the DSPE-PEG induced vinorelbine leakage.

However, at high vinorelbine/HSPC ratio, the vinorelbine loading into 5-ssa liposomes with a high PEG grafting density was also markedly affected. To develop pegylated liposomal vinorelbine formulations with stable encapsulation, "post-insertion" technology was adopted. The incubation of DSPE-PEG polymeric micellar solution with vinorelbine-loaded HSPC/chol vesicles resulted in ~100% insertion of DSPE-PEG molecules into outer lipid monolayers. Moreover, even for the vesicles with entrapped anions that could not mediate effective and stable vinorelbine encapsulation in the presence of peglipids, the insertion of peglipids only induced slight drug release.

Perhaps, the direct interaction of vinorelbine with peglipids was prevented when only the outer monolayer was modified with peglipids; thus resulting in more stable vinorelbine loading. Using "post-insertion" technology, the in vitro stability of pegylated liposomal vinorelbine was also markedly improved.

To further prove the advantages of "post-insertion", the in vitro release studies were performed in ammonium-containing medium. The formulations were prepared using different pegylation technology, but molar percent of peglipids in the outer monolayer was equivalent. As expected (Cattel et al., 2003; Siegal et al., 1995; Takeuchi et al., 2001; Vail et al., 2002), increasing PEG grafting density induced accelerated drug release, but at the same outer monolayer PEG grafting density, "post-insertion" formulations were still more able to improve the retention of vinorelbine.

In plasma, high PEG content formulations could circulate more time because they are more able to evade RES, which might counteract the effects of DSPE-PEG on drug release, resulting in prolonged circulation halftime of entrapped drugs. However, for "co-dissolving" formulations, increasing peglipids did not prolong the half-lives of total vinorelbine, indicating the occurrence of considerable leakage in circulation compared to "post-insertion" formulations.

Slow release formulations might be advantageous since they could deliver more drugs to tumor providing that the same amount of vesicles could accumulate into malignant zones. As a cell cycle-specific drug, vinorelbine was more suited to be delivered by slow release delivery systems. The slow release of vinorelbine could prolong the exposure time of tumor cells to drugs, during which non-mitotic cells might enter mitosis phase, thus becoming sensitive to vinorelbine.

Our results also proved this. In L1210 ascitic tumor model, "post-insertion" formulation with ~0.5 mol% peglipids were the most therapeutically active. Subsequently, the antitumor effects of this formulation were tested in the other two tumor models (RM-1 and S-180). Again, it was proved that this formulation was more efficacious than conventional liposomes and free vinorelbine.

In all, a novel method was developed that could be employed to prepare "stealth" formulations of vinorelbine. Compared to conventional methods, this method could reduce the effects of peglipids on vinorelbine loading, release and retention. Moreover, a promising formulation was discovered, which had the enhanced antitumor effects and was worthy of further evaluation.

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