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Research Paper

Encapsulation of vinorelbine into cholesterol-polyethylene glycol coated vesicles: drug loading and pharmacokinetic studies

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Abstract

Objectives Pegylated liposome formulations of vinorelbine with prolonged circulation half-life ($t^1/_2$) are desirable. However, DSPE-PEG could affect vinorelbine loading into vesicles due to electrostatic interactions. To resolve this problem, chol-PEG was used to prepare pegylated liposomal vinorelbine and the factors affecting drug loading and plasma pharmacokinetics were investigated.

Methods Vinorelbine was loaded into liposomes using a novel triethylamine 5-sulfosalicylate gradient. The effects of cholesterol and chol-PEG on drug loading were investigated. Pharmacokinetic studies were performed in normal KunMing mice treated with different liposomal vinorelbine formulations. To clarify the effects of chol-PEG on membrane permeability, drug release experiments were performed based on the fluorescence dequenching phenomenon of a fluorescence marker.

Key findings In contrast to DSPE-PEG, even at high PEG grafting density (~8.3 mol%), chol-PEG had no effect on vinorelbine loading into HSPC/cholesterol (3 : 1, mass ratio) vesicles. However, for the formulations with low cholesterol content (HSPC/cholesterol 4 : 1), loading efficiency decreased with increasing chol-PEG content. *In vivo*, the vinorelbine $t^1/_2$ of low cholesterol formulations decreased with increasing chol-PEG content, but for high cholesterol liposomes, the maximum vinorelbine $t^1/_2$ was achieved at ~3 mol% chol-PEG grafting density. The resulting vinorelbine circulation $t^1/_2$ was ~9.47 h, which was greater than that of non-pegylated liposomes (~5.55 h). Drug release experiments revealed that chol-PEG might induce membrane defects and concomitant release of entrapped marker, especially at high chol-PEG density.

Conclusions Through the investigation of the effects of chol-PEG and cholesterol, an optimum pegylated liposomal vinorelbine formulation with prolonged $t^1/_2$ was achieved. In plasma, the membrane defect induced by chol-PEG may counteract the long circulation characteristics that chol-PEG afforded. When these two opposite effects reached equilibrium, the maximum vinorelbine $t^1/_2$ was achieved.

Keywords cholesterol-polyethylene glycol; drug loading; liposomes; pharmacokinetics; vinorelbine

Introduction

Liposomes can be used to deliver various types of drugs, resulting in many therapeutic advantages including prolonged action, increased bioavailability, improved safety and enhanced efficacy.[1–3] As carriers of antineoplastic drugs, liposomes could alter the pharmacokinetics and biodistribution of entrapped drugs, thus improving therapeutic indices.[4] The passive targeting of liposomes is associated with the physiological properties of tumours.[5] Due to their rapid growth, tumours usually have leaky blood vessels with pore sizes ranging from 100 to 780 nm. In contrast, the opening of normal blood vessels is approximately 2 nm.[4,5] Therefore, if liposomes have a relatively small vesicle size, long circulation time and stable drug encapsulation, they could passively accumulate in tumour zones.

Although conventional (non-pegylated) liposomes could improve the safety and efficacy of antineoplastic drugs to a certain degree, they are easily recognised by the

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reticuloendothelial system. Therefore, following intravenous injection, a large proportion of liposomes may be cleared from the circulation before they reach the tumour zone.[6] To resolve this problem, stealth technology was invented in the late 1990s.[6,7] Because their surface was coated with hydrophilic polymers such as the polyethylene glycol (PEG) polymer, stealth liposomes could effectively evade recognition and clearance by the reticuloendothelial system, thus resulting in long circulation time and high passive targeting efficiency.[7–9] Furthermore, modification of vesicle surface with PEG polymer could prevent the aggregation of vesicles and improve long-term stability.[6] Therefore, stealth liposome formulations are advantageous compared with conventional liposomes.

Vinorelbine is a semisynthetic vinca alkaloid with antitumour activity that can inhibit mitotic microtubule formation, thus inducing a blockade of cells at metaphase.[10] Although it possesses relative selectivity for mitotic microtubules compared with other vinca alkaloids, vinorelbine still has clinical side-effects such as granulocytopenia, leucopenia and anaemia.[11] Therefore, it is desirable to prepare vinorelbine liposomal formulations to improve its safety and efficacy.[4,12–18] To this end, liposomal vinorelbine formulations with slow drug release rates are preferable since this type of formulation could deliver more of the drug to tumours and increase the exposure time of tumour cells to the drug. However, it is difficult to develop liposome formulations of vinorelbine, especially stealth formulations, due to its lipophilicity, membrane permeability and potential interaction with PEG lipids.[13,14] Typically, to prepare stealth liposomes, negatively charged DSPE-PEG is used. However, it has been reported that DSPE-PEG could induce accelerated release of vincristine from vesicles.[16,19] Since vinorelbine is structurally similar to vincristine, a similar phenomenon might occur when vinorelbine is loaded into vesicles grafted with DSPE-PEG molecules.

To investigate the effects of PEG lipids on drug loading, vinorelbine was loaded into non-pegylated and pegylated vesicles with high cholesterol content using a novel triethylamine 5-sulfosalicylate gradient. Upon using vesicles made of DSPE-PEG, the loading efficiency significantly decreased. However, chol-PEG had no influence on vinorelbine loading. Therefore, we focused on the encapsulation of vinorelbine into chol-PEG coated liposomes and investigated the factors affecting drug loading and plasma pharmacokinetics. A fluorescence dequenching method was used to investigate the effects of chol-PEG on membrane permeability. Irinotecan, a fluorescent antineoplastic agent that is weakly basic, was entrapped into chol-PEG liposomes as a marker. Since following irinotecan release the quenched fluorescence could be regenerated, the drug release rate could be rapidly evaluated. This study is the first attempt to prepare pegylated liposomal vinorelbine using chol-PEG and a novel ion gradient was developed to stabilise the entrapped vinorelbine.

Materials and Methods

Materials

KunMing (KM) mice (8–10 weeks old) were obtained from Hebei Medical University, China. 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 (mPEG$_{2000}$-DSPE) was obtained from Genzyme Pharmaceuticals (Liestal, Switzerland). Poly(oxy-1,2-ethanediyl),α-(3β)-cholest-5-en-3yl, ω-hydroxy-(cholesterol-PEG$_{2000}$) was purchased from NOF Corporation (Tokyo, Japan). Cholesterol, Sepharose 4B and Sephadex G-75 (medium) were obtained from Sigma Chemical Company (St Louis, MO, USA). Nucleopore polycarbonate filters (47 mm, 0.1 μm pore size) were obtained from Northernlipids, Inc. (Burnaby, Canada). All other chemicals (including triethylamine and 5-sulfosalicylic acid) used in this study were of analytical or high-performance liquid chromatography (HPLC) grade. The chemical structures of materials are presented in Figure 1.

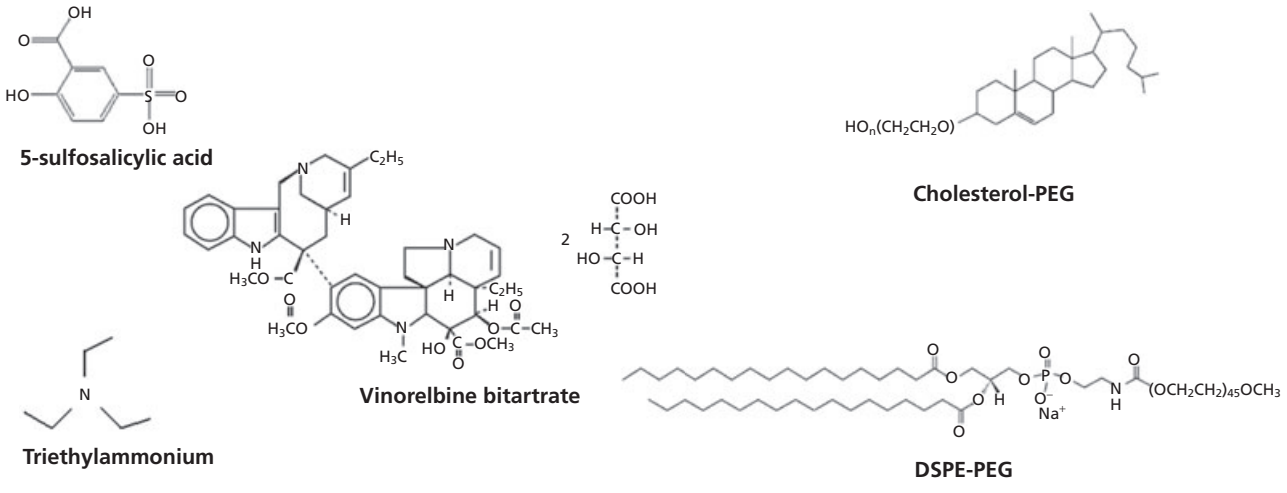

Figure 1 Chemical structures of 5-sulfosalicylic acid, triethylamine, vinorelbine bitartrate, chol-PEG and DSPE-PEG.

Preparation of liposomes

Liposomes were prepared according to the following procedure. Briefly, mixtures of HSPC, cholesterol and $mPEG_{2000}$-DSPE (or chol-PEG) were solubilised 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. The dried lipid films were subsequently hydrated with 300 mM triethylamine salt of 5-sulfosalicylic acid. The hydration process was performed at 60°C for 1 h. The dispersion was extruded eight times through polycarbonate filters (0.10 μm) using 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 analysed using quasi-elastic light scattering (Zetasizer Nano ZS; Malvern Instruments, Worcestershire, UK). Before analysis, the samples were diluted in 0.9% NaCl with a volume ratio of 1 : 200. DTS4.0 software was used to collect the data and analysis was done using multinarrow modes (Malvern Instruments, Worcestershire, UK).

Remote loading of liposomes

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

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 a Sephadex G-75 mini-column (56 mm × 8 mm i.d.), and then eluted using 0.9% NaCl solution.

Liposomal formulations

For all the formulations used in the pharmacokinetic studies, the drug to HSPC mass ratio was 0.3, and ~100% vinorelbine was loaded into vesicles.

Pharmacokinetic studies

Plasma pharmacokinetic analysis was performed in normal KM mice. All animal experimental protocols were approved by the Institutional Animal Care and Use Committee (ZhongQi Pharmaceutical Technology co., Ltd) and complied with the code of ethics for animal experiments.

For pharmacokinetic studies, KM mice received injections of 10 mg/kg vinorelbine bitartrate in a single dose of liposomal vinorelbine formulations via the right lateral tail vein. At the indicated time points, blood samples were obtained via cardiac puncture under anaesthesia and were collected in Eppendorf tubes containing sodium heparin as an anticoagulant. Blood samples were centrifuged at 600*g* for 10 min to separate the plasma. The plasma samples were stored at −20°C until analysis.

Vinorelbine concentrations in plasma samples were determined by HPLC. To 20 μl plasma, 20 μl purified water and 460 μl methanol were 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 (Waters Corporation, Massachusetts, USA) controlled by Millennium 32 software was used for chromatographic analysis (2690 liquid chromatograph and 996 diode array detector). The HPLC separations were achieved using a Zorbax C_{18}, 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 the 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 had an r value of 0.999. The pharmacokinetic variables were calculated using DAS 2.0 software (Mathematical Pharmacology Professional Committee of China, Shanghai, China).

In-vitro release studies

Irinotecan release from different chol-PEG vesicles was monitored using a fluorescence dequenching assay. Before analysis, an aliquot of liposomal irinotecan was diluted 1000 fold by injection into a quartz cuvette containing a solution of glucose/histidine/NH_4Cl (250 mM/10 mM/20 mM, pH 7.5). A Hitachi F-4600 fluorescence spectrophotometer was used (Hitachi, Tokyo, Japan). The fluorescence intensity data were collected continually with a time interval of ~8 s. The excitation and emission wavelengths were 381 and 420 nm, respectively. Temperature was controlled at 37°C using a jacketed sample holder connected to a circulating water bath. The intensity data were converted to concentration data according to the fluorescence intensity versus concentration standard curve. The percentage release was determined by: $100 \times ([\text{irino}]_t - [\text{irino}]_0) / [\text{irino}]_{\text{total}}$, where $[\text{irino}]_t$ and $[\text{irino}]_0$ are free irinotecan concentrations at time points t and 0, and $[\text{irino}]_{\text{total}}$ is the total irinotecan concentration, including both free and liposomal irinotecan. To quantitatively compare the difference in drug release kinetics, the percentage release was plotted as a function of time.

Statistical analysis

The results in all figures and tables are shown as the mean ± SD. The differences in plasma vinorelbine concentrations were examined using the Kruskal-Wallis test at each time point. Post-hoc comparison of the means of individual treatments was performed using Nemenyi's test. In all cases, $P \leq 0.05$ was considered to be statistically significant.

Results

Drug loading

Since vinorelbine is lipophilic and membrane-permeable relative to other vinca alkaloids, it is difficult to obtain highly stable liposomal vinorelbine formulations using current drug loading techniques. To resolve this problem, we developed a

Table 1 Effects of drug/lipid ratio, PEG lipids and PEG grafting density on drug loading

Formulations	% Loading at different drug/lipid mass ratios (from 0.3 to 0.6)			
	0.3	0.4	0.5	0.6
Without PEG lipids	100 ± 0.7	100 ± 0.3	99.5 ± 0.8	96.3 ± 1.2
8.3% DSPE-PEG	14.7 ± 0.6	8.4 ± 1.2	6.7 ± 0.9	6.9 ± 1.3
0.5% chol-PEG	99.0 ± 1.4	98.7 ± 2.1	ND	96.7 ± 1.0
2.9% chol-PEG	98.6 ± 1.3	98.4 ± 1.5	97.8 ± 1.3	98.1 ± 0.4
8.3% chol-PEG	99.3 ± 0.9	98.8 ± 2.0	98.7 ± 1.7	96.3 ± 1.9

Vinorelbine was loaded into non-pegylated vesicles or pegylated vesicles modified with DSPE-PEG (8.3%) or chol-PEG at different PEG grafting density (0.5, 2.9 and 8.3%) using a triethylamine 5-sulfosalicylate gradient. In all cases, the HSPC/cholesterol mass ratio was 3 : 1. PEG grafting density referred to the molar percent of DSPE-PEG (or chol-PEG) relative to HSPC. Data represent the mean ± SD of three independent experiments. ND, not determined.

Table 2 Effects of cholesterol content and PEG grafting density on drug loading

HSPC/cholesterol (mass ratio)	% Loading at different mol% chol-PEG						
	1	2	3	4	5	6	7
6 : 1	–	–	2.5 ± 1.3	–	–	–	–
4 : 1	99.5 ± 1.7	–	95.5 ± 2.0	–	92.9 ± 0.9	–	70.7 ± 0.8
3 : 1	–	99.4 ± 0.6	99.7 ± 1.4	99.6 ± 2.1	99.5 ± 2.2	99.7 ± 1.5	99.8 ± 1.2

Vinorelbine was loaded into HSPC/cholesterol vesicles with different cholesterol content (HSPC/cholesterol 6 : 1 to 3 : 1, mass ratio) and different PEG grafting density (ranging from 1 to 7%) using a triethylamine 5-sulfosalicylate gradient. PEG grafting density refers to the molar percent of chol-PEG relative to HSPC. Data represent the mean ± SD of three independent experiments.

novel intraliposomal stabilisation strategy, which involved the use of 5-sulfosalicylic acid as entrapping agent in conjunction with a triethylamine gradient.

As shown in Table 1, the triethylamine 5-sulfosalicylate gradient could mediate effective and stable vinorelbine loading into conventional HSPC/cholesterol (3 : 1, mass ratio) liposomes. However, when the vesicles were grafted with ~8.3 mol% DSPE-PEG (both sides), the loading efficiency was considerably decreased to <20%. In contrast, the modification of the vesicles with the same amount of chol-PEG had no effect on drug loading. When the drug-to-lipid mass ratio ranged from 0.1 to 0.6%, loading of vinorelbine remained almost constant, with a value of >95%. Thus, chol-PEG was more suitable to prepare stealth liposomes than DSPE-PEG because it does not interact with vinorelbine.

We investigated the effects of drug-to-lipid ratio and chol-PEG content on vinorelbine loading. In this series of experiments, the HSPC/cholesterol mass ratio was set at 3 : 1, corresponding to ~ 80% of the maximum cholesterol content that the liposome bilayer could accommodate. The molar percent of chol-PEG (relative to HSPC) was 0.5, 2.9 or 8.3%, corresponding to three different PEG polymer conformation regimes, namely, interdigitated mushroom, mushroom and brush conformations.[5,20] Interestingly, it was found that under the current conditions, both factors had almost no influence on drug loading and for all the formulations loading efficiencies were ~100%.

It was found that cholesterol considerably affected vinorelbine loading (Table 2). When the HSPC/cholesterol mass ratio was reduced to 6 : 1, only ~2.5% vinorelbine was loaded into vesicles coated with ~3% chol-PEG. At a HSPC/cholesterol mass ratio of 4 : 1, the loading efficiency decreased with increasing chol-PEG grafting density. For example, when the HSPC/cholesterol 4 : 1 vesicles were coated with ~7% chol-PEG, only ~70.7% vinorelbine loading could be achieved. However, when vesicles with high cholesterol content were used, chol-PEG did not influence vinorelbine loading at all. Even though at some ratios only limited experiments were performed, the effects of chol-PEG could still be clearly observed.

Pharmacokinetic studies

Three pharmacokinetic experiments were performed to investigate the in-vivo behaviour of liposomes coated with chol-PEG. First, the effects of chol-PEG content on plasma pharmacokinetics of high cholesterol vesicles were evaluated. In this experiment, all the vesicles had a mean size of ~100 nm, ~ 100% vinorelbine loading and a HSPC/cholesterol mass ratio of 3 : 1. As shown in Figure 2a, increased chol-PEG grafting density did not increase the half-life ($t^1/_2$) correspondingly; the longest $t^1/_2$ was achieved at a chol-PEG content of ~3% ($P \leq 0.05$). In contrast, when the cholesterol content in the lipid bilayer decreased (HSPC/cholesterol 4 : 1), chol-PEG exerted its influence in a different manner. As shown in Figure 2b, the $t^1/_2$ values of liposomal vinorelbine decreased with increasing chol-PEG content. When 1% chol-PEG was incorporated into the lipid membrane, the resulting $t^1/_2$ was 4.85 h. However, the elevation of chol-PEG content to 5% led to the deduction in both $t^1/_2$ and plasma concentration. The Kruskal-Wallis test revealed that differences among these three formulations were significant ($P \leq 0.05$, $t = 4$ h). Therefore, it seems that high cholesterol

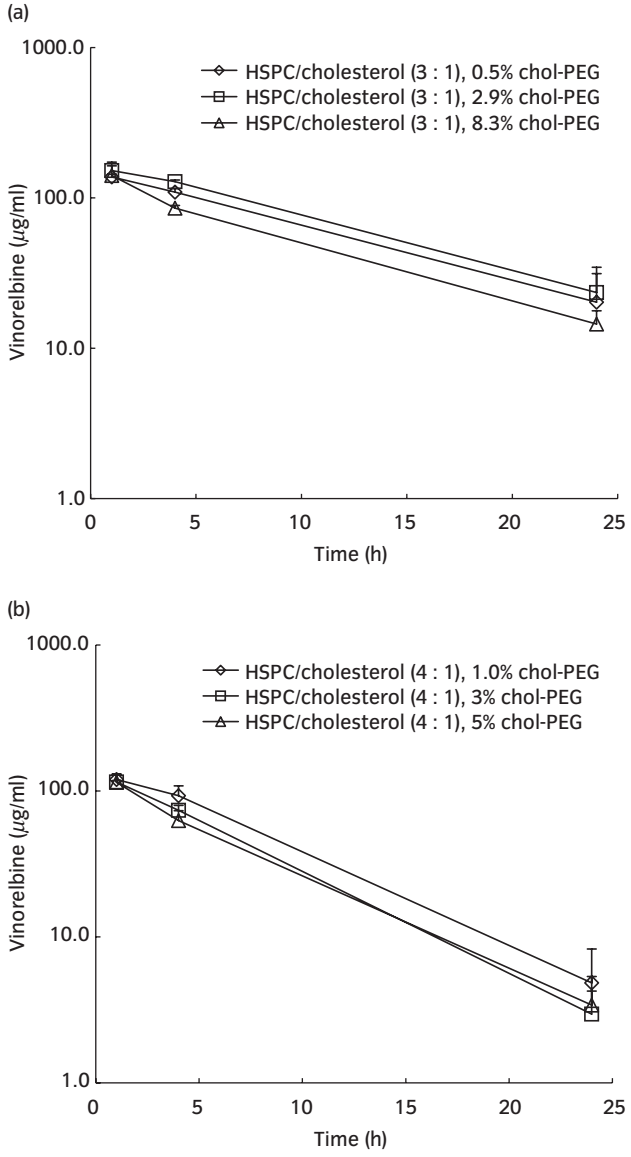

Figure 2 Effects of HSPC/cholesterol ratio and chol-PEG content on plasma pharmacokinetics. All liposomal vinorelbine formulations were administrated to KunMing mice at 10 mg vinorelbine bitartrate/kg via the right lateral tail vein ($n = 3$). Data points represent the mean vinorelbine concentration ± SD. (a) HSPC/cholesterol (3 : 1, mass ratio) vesicles with different chol-PEG grafting density. (b) HSPC/cholesterol (4 : 1, mass ratio) vesicles with different chol-PEG grafting density.

formulations had more stable vinorelbine encapsulation in plasma, which was in agreement with the drug loading experiments.

Given that for liposomal formulations with high cholesterol content a relatively large $t^1/_2$ value of liposomal vinorelbine was not found at the extreme chol-PEG content, chol-PEG must be able to elicit two opposite effects, which could affect liposomal vinorelbine kinetics in different ways. If these two effects could reach equilibrium, the longest retention time of vinorelbine should be achieved. To prove this, a screening experiment was performed, in which grafting density of chol-PEG ranged from 1 to 7%. Based on data from regression analysis, it was found that when ~3% chol-PEG was incorporated in the lipid bilayer, liposomal vinorelbine had the longest circulation $t^1/_2$ of vinorelbine (Table 3). Although the calculated $t^1/_2$ from 6 to 7 mol% grafting density of chol-PEG increased slightly, the statistical difference was not significant.

As the high cholesterol formulation with ~3% chol-PEG possessed the longest circulation time, it was chosen for further investigation. Compared with non-pegylated liposome formulations, this chol-PEG liposome formulation could considerably prolong the $t^1/_2$ of vinorelbine (Figure 3). The resulting $t^1/_2$ values for both formulations were 9.47 and 5.55 h, respectively, indicating a significant improvement. Statistical analysis proved that the difference between these two formulations was significant ($P \leq 0.05$).

Effects of chol-PEG on the release of entrapped marker

Since elevation of the chol-PEG content did not lead to prolonged circulation $t^1/_2$ of vinorelbine, we suspected that chol-PEG might induce accelerated drug release due to membrane defects. To test this, irinotecan was loaded into chol-PEG modified liposomes using the triethylamine 5-sulfosalicylate gradient. At a drug-to-liposome mass ratio of 0.2 (irinotecan/HSPC), after incubation at 60°C for ~40 min, ~100% of irinotecan was loaded into the vesicles, irrespective of the chol-PEG content (2–7 mol% of HSPC), indicating no evident interactions between irinotecan and chol-PEG. Inside the vesicles, the fluorescence of irinotecan quenched due to the high irinotecan concentration. However, when irinotecan was released into the release buffer, dequenching could occur. Accordingly, a fluorescence dequenching method was developed to monitor irinotecan release and evaluate the influence of chol-PEG on membrane permeability.

The release experiments were performed in isotonic and NH_3-containing buffer at body temperature. Free ammonium could freely permeate the lipid bilayer, thus reversing the pH gradient and triggering drug release. Moreover, because the samples were diluted into release buffer in a 1: 1000 volume ratio, the 'sink' status could be created, which permitted sustained drug release from vesicles. Both free ammonium and the 'dilution effect' could drive drug release and the difference in drug release could be determined in a short time.

As shown in Figure 4, the irinotecan release rate from vesicles increased with increasing chol-PEG grafting density. Since, with the exception chol-PEG content, all the formulation properties were the same, the difference in irinotecan release could be associated with the grafted chol-PEG. Therefore, chol-PEG might affect membrane permeability, thus inducing membrane defects and concomitant drug release. Perhaps, in the same manner, chol-PEG induced accelerated vinorelbine release, thus counteracting the long circulating effects endowed by it.

Discussion

In this study, we focused on the use of chol-PEG to prepare stealth liposomes. The factors affecting drug loading, release and plasma pharmacokinetics were carefully examined. There

Table 3 Plasma vinorelbine concentration after injection of vesicles with different chol-PEG content

chol-PEG (mol%)	Plasma vinorelbine concentration (μg/ml) at different times (h)			Calculated half-life (h)
	1	4	24	
2	212 ± 22.4	140 ± 15.2	22.0 ± 5.7	7.20
3	194 ± 34.4	111 ± 29.3	28.8 ± 7.5	8.98
4	193 ± 19.3	123 ± 18.4	25.2 ± 5.7	8.14
5	178 ± 11.8	110 ± 10.6	17.9 ± 3.7	7.17
6	164 ± 18.9	116 ± 5.5	18.5 ± 3.9	7.14
7	160 ± 10.3	130 ± 11.0	20.1 ± 2.9	7.58

All liposomal vinorelbine formulations were administrated to KunMing mice at 10 mg vinorelbine/kg via the tail vein ($n = 3$). Data points represent the mean vinorelbine concentration ± SD. In all cases, the vesicles had a mean size of ~100 nm, drug/lipid mass ratio of 0.3, and ~100% loading. PEG grafting density refers to the molar percent of chol-PEG relative to HSPC and HSPC/cholesterol mass ratio was 3 : 1.

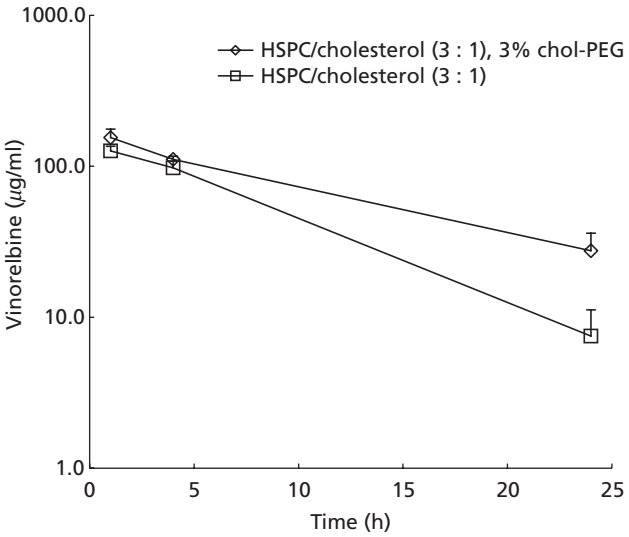

Figure 3 Plasma pharmacokinetics of pegylated and conventional liposomes. Both liposomal vinorelbine formulations were administrated to KunMing mice at 10 mg vinorelbine bitartrate/kg via the right lateral tail vein ($n = 3$). Data points represent the mean vinorelbine concentration ± SD. Except that the pegylated liposomal formulation was modified with 3% chol-PEG, all other formulation properties were the same for both formulations.

are three main points of interest. The first concerns why chol-PEG did not affect vinorelbine loading in a manner similar to DSPE-PEG, the second concerns the key role of cholesterol in drug loading, and the third concerns why the longest vinorelbine circulation time could not be achieved by elevation of the chol-PEG content in the lipid bilayers.

To load vinorelbine into vesicles, an active loading technology was used (Figure 5), which was similar to the traditional ammonium sulfate gradient method.[21,22] In this method, the dried lipid membrane was hydrated with triethylamine/5-sulfosalicylic acid solution to form triethylamine/5-sulfosalicylic acid containing liposomes. Following extrusion to reduce vesicle size, the liposomes were suspended in neutral sucrose/histidine buffer and thus a transmembrane triethylamine/5-sulfosalicylic acid gradient was generated. Because triethylamine is a weak base (pKa = 10.75) and 5-sulfosalicylic acid is a strong acid ($pKa_1 \leq 1$ and $pKa_2 = 2.85$),[23] triethylamine would dissociate to produce proton and neutral triethylamine that could freely permeate the lipid bilyer. Therefore, the presence of the triethylamine/5-sulfosalicylic acid gradient means the presence of a neutral triethylamine gradient, which could induce the movement of neutral triethylamine towards the extraliposomal medium. The transmembrane transportation of neutral triethylamine results in the acidification of intraliposomal media, which drives the intraliposomal accumulation of neutral vinorelbine molecules. In the internal acidic medium, vinorelbine becomes protonated and might be precipitated by 5-sulfosalicylic acid anions, which further promotes more neutral vinorelbine uptake into the vesicles. Based on this drug loading mechanism, only free neutral drug molecules could permeate lipid bilayers and the tartrate anions could not be loaded into the vesicles. This hypothesis is rational because it is difficult for protonated molecules to permeate the membranes due to their hydrophilicity. The factors that could induce the generation of a transmembrane concentration gradient of neutral drugs (e.g. protonation and precipitation) are the driving forces of drug loading and the presence of a substantial amount of neutral drug molecules outside the vesicles is the essential condition for drug loading.

Similar to other vinca alkaloids, vinorelbine has pKa values of ~5.0 and 7.4,[13,14] and therefore in the external media (pH 7.5), ~50% vinorelbine carried one positive charge (Handerson-Hasselbalch equation). In fact, because upon drug loading, vinorelbine bitartrate was used, the external pH slightly decreased and more vinorelbine might be positively charged. Since DSPE-PEG is negatively charged (Figure 1) (it carries one negative charge on the phosphate), it can bind positively charged vinorelbine and reduce the amount of neutral vinorelbine in the outer medium due to the shifting of dissociation equilibrium. If the vesicle surface is completely covered by DSPE-PEG, a negatively charged barrier will be generated and vinorelbine will be absorbed on the vesicle surface instead of loading into the vesicles. Therefore, at high DSPE-PEG grafting density (8.3 mol%, corresponding to brush PEG conformation),[5,20] only a limited amount of vinorelbine was loaded into the vesicles. In contrast to DSPE-PEG, chol-PEG is electrically neutral and cannot interact with vinorelbine via electrostatic interactions, and thus exerts no influence on vinorelbine loading.

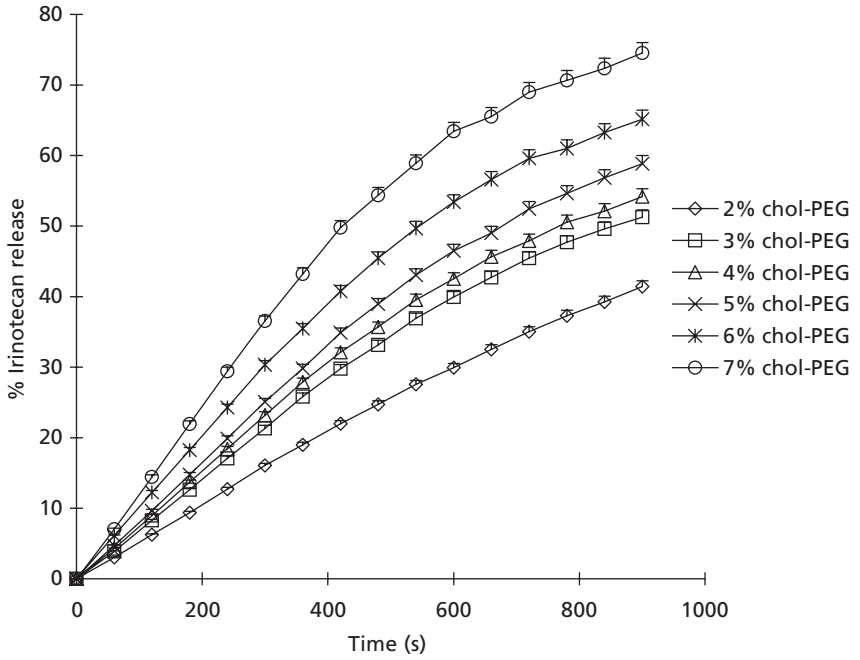

Figure 4 Effects of chol-PEG on the release of entrapped marker. The fluorescence marker, irinotecan, was loaded into chol-PEG modified liposomes using a triethylamine 5-sulfosalicylate gradient. All the formulations had a drug/lipid mass ratio of ~0.2, and ~100% encapsulation, irrespective of chol-PEG content. Irinotecan release from vesicles was monitored using a fluorescence dequenching assay. To compare the difference in drug release kinetics, fluorescence intensity data were converted to % drug release data, which were plotted as a function of time.

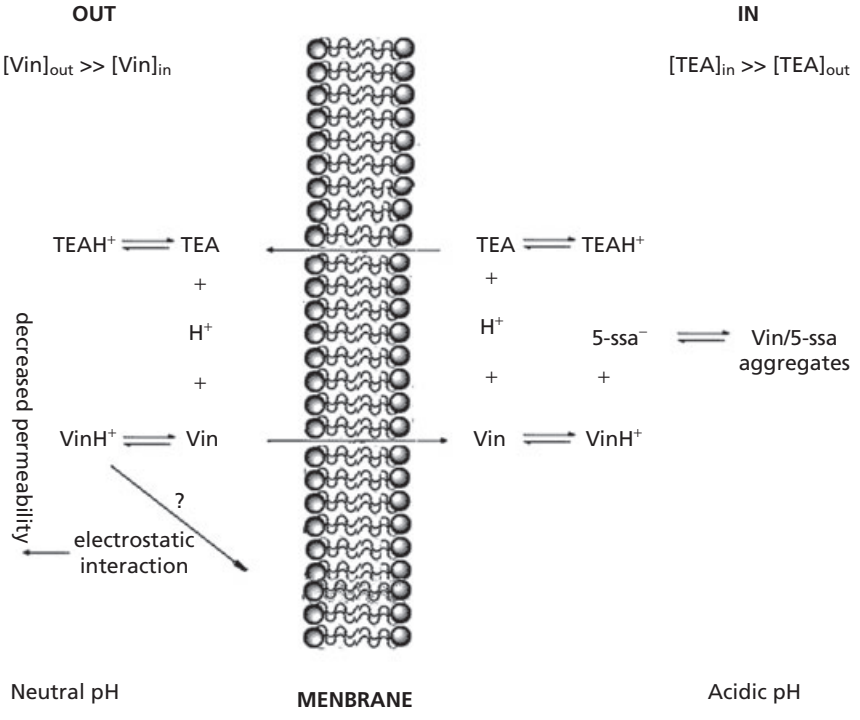

Figure 5 Schematic representation of vinorelbine loading into vesicles in response to triethylamine salt of 5-sulfosalicylic acid gradient. 5-ssa, 5-sulfosalicylic acid; TEA, triethylamine; Vin, vinorelbine.

Cholesterol content is a key factor influencing drug loading. When the lipid bilayer contained a relatively small amount of cholesterol (HSPC/cholesterol 6 : 1), almost no vinorelbine could be loaded into the vesicles. To explain this phenomenon, the drug loading procedure should be taken into account. The drug loading technology used in this study is also essentially a type of pH gradient method despite that it adopts a self-generating system.[21,22] This method relies on the incubation of drugs and liposomes exhibiting a transmembrane triethylamine/5-sulfosalicylic acid gradient at a temperature above the phase transition temperature of the bulk phospholipids to promote drug loading. It has been reported that when liposomes contain <30 mol% cholesterol, the formulation will become extremely sensitive to temperature changes, and at high temperatures, grain boundaries, lipid domains and disk-like structures will be formed, leading to the collapse of the pH gradient and unstable drug loading.[24,25] Perhaps for this reason, the low cholesterol formulation mediated almost no drug encapsulation. When the cholesterol content was increased to ~33 mol% (HSPC/cholesterol 4 : 1), efficient loading could be achieved, but now the loading became sensitive to chol-PEG content. This might be associated with the physiochemical properties of chol-PEG. Chol-PEG is an amphiphilic molecule, which can form polymeric micelles in aqueous medium and solubilise hydrophobic lipids, thus affecting the formation and permeability of lipid bilayers.[26] To what degree the lipid bilayer is affected depends on both cholesterol and chol-PEG content. For liposomes with high cholesterol content, the membrane has decreased permeability, so chol-PEG had almost no effects on vinorelbine loading.

On the vesicle surface, grafted PEG polymers might exhibit different conformations. The conformations of PEG molecules depend on PEG grafting density and PEG molecular weight, and can be divided into three regimes, namely, interdigitated mushroom, mushroom and brush conformations.[5,20] As a rule, high PEG grafting density (e.g. 8.3%) could result in complete coverage of the vesicle surface and the brush conformation. Accordingly, a steric barrier will be formed around the vesicles, which could effectively prevent the adhesion of lipoproteins and recognition by the reticuloendothelial system, thus leading to extended circulation time.[7,9,27] When we employed DSPE-PEG to prepare the stealth liposomes (e.g. pegylated liposomal mitoxantrone), it was found that the circulation time increased with increased PEG grafting density, where DSPE-PEG content ranged from 0.5 to 8.3%.[28] However, this was not the case when chol-PEG was used. Based on our experimental data, the maximum $t^1/_2$ could be achieved only when the PEG grafting density was ~3%; further increasing the chol-PEG content caused a decreased plasma circulation time of liposomal vinorelbine. To explain this phenomenon, the difference between chol-PEG and DSPE-PEG must be considered. It has been reported that since the cholesterol anchor could be located deeper in the liposome membrane, the PEG chain of chol-PEG exhibits a minimum conformational flexibility relative to those of other PEG-lipid derivatives, thus resulting in lower efficiency to prolong the circulation time of liposomes.[6,26,29–32] Moreover, chol-PEG increases liposomal membrane fluidity and permeability as a result of structural perturbations of the host bilayer due to the presence of the large PEG moiety and no OH group in position 3 of cholesterol.[6,26,29–32] Perhaps increased chol-PEG grafting density can induce two opposite effects. First, the opsonisation of the liposome surface with complement proteins might be reduced with increasing PEG density, thus leading to long vesicle circulation time. Second, increased amount of chol-PEG grafted in the lipid bilayer might induce membrane defects and concomitant vinorelbine release. The first effect has been well documented[7,9,27] and in-vitro release experiments using irinotecan as a marker proved the existence of the second effect. At a certain chol-PEG content, these two opposite effects reach equilibrium, leading to the maximum $t^1/_2$ of total plasma vinorelbine. Presumably, because of above reasons, it is not possible to achieve the longest circulation time only by increasing chol-PEG content.

Conclusions

In this study, vinorelbine was loaded into vesicles in response to a novel triethylamine 5-sulfosalicylate gradient. Drug loading experiments revealed that unlike DSPE-PEG, chol-PEG did not affect the uptake of vinorelbine into liposomes with high cholesterol content despite the fact that it affected drug loading into low cholesterol formulations. *In vivo*, the vinorelbine $t^1/_2$ of low cholesterol formulations decreased with increasing chol-PEG content, but for high cholesterol liposomes the maximum vinorelbine retention time was achieved at a PEG grafting density of ~3 mol%. The optimal chol-PEG liposomes exhibited markedly prolonged vinorelbine $t^1/_2$ compared with conventional liposomes, indicating the effectiveness of chol-PEG for the preparation of stealth liposomes. Our results also demonstrated that chol-PEG might perturb the lipid bilayer, resulting in increased membrane permeability and concomitant drug release. The design of novel cholesterol-PEG conjugates, in which cholesterol and PEG are conjugated with a flexible linker, might resolve this problem.

Declarations

Conflict of interest

The Author(s) declare(s) that they have no conflicts of interest to disclose.

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