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Research Paper

Novel sulfobutyl ether cyclodextrin gradient leads to highly active liposomal irinotecan formulation

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Abstract

Objectives Liposomal delivery of irinotecan could provide protection against drug hydrolysis, deliver more active lactone form to tumours and prolong irinotecan exposure time. Nevertheless, conventional drug-loading technologies have typically resulted in undesired drug retention properties. To resolve the problem, a modified gradient loading method was developed and the resulting formulations were evaluated in a systemic manner.

Methods Irinotecan was loaded into liposomes using a novel sulfobutyl ether beta-cyclodextrin (sbe-CD) gradient. The effect of drug-to-lipid ratio (D/L) and polyethylene glycol (PEG) grafting density were investigated. Drug release experiments were performed in ammonium-containing medium based on the fluorescence dequenching phenomenon of irinotecan. Pharmacokinetic studies were performed in normal balb/c mice treated with different formulations. To compare the anti-tumour effect of different formulations, an RM-1 prostate cancer model was used. Acute toxicity studies were performed in healthy female c57 mice.

Key findings Irinotecan could be encapsulated into liposomes with > 90% loading efficiency at a high drug-to-lipid mass ratio (> 0.5). In-vitro release experiments revealed that sbe-CD anion was more able to retain irinotecan than sulfate. Moreover, the elevated D/L ratio elicited decreased drug release kinetics. Both trends had also been observed when the effects of anions and D/L ratio on half-life of irinotecan were assessed. Pegylated liposomal irinotecan loaded with sbe-CD/triethylammonium gradient had irinotecan half-life values ranging from 9.4 to 13.1 h, surpassing vesicles prepared by the triethylammonium sulfate method (~4.5 h). In the RM-1 tumour model, all the liposomal irinotecan formulations were more therapeutically active than free irinotecan and the formulation with a high D/L ratio was the most efficacious. Moreover, the high D/L formulation might be less toxic than free irinotecan based on acute toxicity studies.

Conclusions The novel sbe-CD gradient could mediate effective irinotecan loading and improve irinotecan retention, thus resulting in highly active liposomal irinotecan formulations. The improvement in drug retention might be associated with the formation of complicated aggregates inside vesicles.

Keywords drug loading; irinotecan; liposomes; sulfobutyl ether cyclodextrin; therapeutic index

Introduction

The camptothecins are a new class of anticancer agent with documented clinical activity in human neoplasms.[1–3] They can interact specifically with topoisomerase I, resulting in the formation of stable topoisomerase I, DNA and camptothecin ternary complexes. Double-stranded DNA damage can be produced during DNA synthesis when replication enzymes interact with the ternary complexes. Since mammalian cells cannot efficiently repair these double-strand breaks, camptothecins can induce severe cytotoxicity, especially in malignant tumour cells with rapid proliferation.[4,5]

Irinotecan is a water-soluble derivative of camptothecin. In the USA, it has been approved for use in solid tumours.[4,6–8] Clinical studies are ongoing to better define its utility

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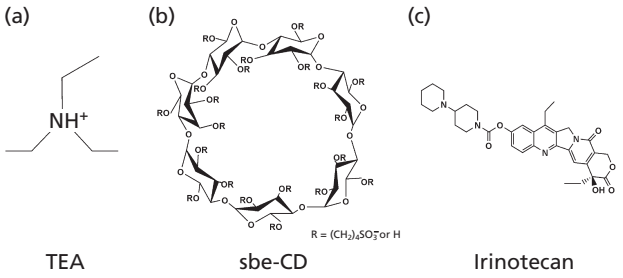

Figure 1 The chemical structures of triethylammonium, sulfobutyl ether cyclodextrin and irinotecan.

and its spectrum of activity in human malignancies.[5,9] As with all camptothecins, irinotecan contains a terminal lactone ring that makes it unstable in aqueous solutions by undergoing a rapid, pH-dependent, non-enzymatic hydrolysis to form an open-ring hydroxyl carboxylic acid, which is a much less potent inhibitor of topoisomerase I.[4,5,8] At neutral or basic pH, the equilibrium for this hydrolysis reaction favours the formation of the less active carboxylate species, while acidic, low pH favours the formation of the active lactone. Following intravenous or oral administration, both the lactone and carboxylate camptothecin forms can be measured in plasma.[4,5,8]

Irinotecan is a weakly basic drug and it can be loaded into vesicles using a pH gradient method.[10–16] In this method, drugs actively accumulate into vesicles in response to the pH gradient. After uptake by vesicles, they might become protonated in the acidic environment of the aqueous core and form specific aggregates with counter ions.[14–16]

Liposome encapsulation could stabilize irinotecan as the active lactone in the acidic internal media.[17–30] Moreover, liposomes could be effectively targeted to malignant zones due to 'enhanced permeability and retention effects', thus leading to improved therapeutic activity.[31] The realization of these advantages relies on the effective retention of irinotecan by vesicles in plasma and its subsequent release at desired sites. However, if traditional pH gradient methods are used,[17–26,28–30] premature drug release might occur, namely, in circulation a substantial amount of irinotecan is released and converted into its less active carboxylate form. Thus, only a limited amount of active lactone is delivered to the tumour, resulting in undesirable safety and efficacy profiles.

In this study, a novel drug loading technology was developed, which involved the use of sulfobutyl ether cyclodextrin (sbe-CD) as an intraliposomal trapping agent in conjunction with transmembrane triethylammonium gradient (Figure 1). Using this method, irinotecan could be effectively loaded into vesicles with high drug load. To screen the optimal formulation, drug release, pharmacokinetics, anti-tumour studies and acute toxicity experiments were performed. This study is a first attempt to load irinotecan using novel sbe-CD gradient and a promising formulation was identified.

Materials and Methods

Materials

Irinotecan hydrochloride was provided by Knowshine Pharmaceuticals, Inc. (Shanghai, 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). Cholesterol (Chol) and Sephadex G-75 (medium) were obtained from the Sigma Chemical Company (St Louis, USA). Nucleopore polycarbonate filters (47 mm, 0.1 μm pore sizes) were obtained from Northernlipids, Inc. (Burnaby, BC, Canada). All other chemicals used in this study were analytical or high-performance liquid chromatography (HPLC) grade.

The RM-1 tumour cell line was originally purchased from Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences (Shanghai, China). Balb/c mice, 8–10 weeks old, were obtained from Hebei Medical University. BDF1 and c57 mice were purchased from Vitalriver (Beijing, China).

Preparation of salts of sulfobutyl ether beta cyclodextrin

A solution of triethylammonium salt of sbe-CD was prepared from commercially achieved sodium salt (CyDex Pharmaceuticals, Inc. Lenexa, USA) by ion-exchange chromatography on Dowex 50W×8-200 resin in the H^+ form, immediately followed by titration with triethylammonium (TEA). Residual sodium was <1% of the cation content as determined by ion chromatography. The TEA concentration was ~0.5 mol/l and the final pH was ~5.5.

Preparation of liposomes

The mixtures of HSPC, Chol and mPEG$_{2000}$-DSPE 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, and the PEG-DSPE/HSPC molar ratio was 0.5, 2.9 and 8.3%, respectively. The dried lipid films were subsequently hydrated with TEA salts of sbe-CD (or 250 mM TEA sulfate). 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. For the comparison, vesicles with entrapped sodium salt of sbe-CD were also prepared using a similar procedure, in which lipid film was hydrated with sodium salt of sbe-CD instead.

The average size of vesicles was analysed 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 50-fold dilution. DTS4.0 software was used to collect the data that were analysed using 'multinarrow modes'.

Remote loading of liposomes

A transmembrane ion gradient was generated across the vesicles by exchanging the extraliposomal buffer using Sephadex G-75 columns. The buffer employed in the experiments was pH 6.5 sucrose/histidine (300/20, mM/mM) buffer.

Upon buffer exchange, empty liposomes with transmembrane ion gradient were mixed with irinotecan at the desired mass ratio. The resulting mixture was incubated at 60°C for the desired time to realize drug loading. For Na sbe-CD-containing vesicles, drug loading was also performed in the presence of nigericin (20 ng/mg HSPC), which was pre-incubated with empty vesicles for 10 min before drug loading.

For determining the loading efficiency, samples of the mixtures were taken and unentrapped irinotecan 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. The loading was calculated using the formula: loading % = liposome fraction/(liposome fraction + free drug fraction) × 100.

Liposome formulations

For performing in-vitro release, pharmacokinetic, acute toxicity and anti-tumour experiments, a series of liposomal formulations were prepared and characterized in accordance with above procedures. The resulting formulations were named pLI-cd-2.9-2, pLI-cd-2.9-3, pLI-cd-2.9-4 and pLI-s-2.9-3, respectively (abbreviations: pLI, pegylated liposomal irinotecan; cd, sulfobutyl ether cyclodextrin; s, sulfate). The number following 'cd' (or 's') refers to the percentage of PEG grafting density. The final number was for drug-to-lipid ratio; if the number was 2, the drug-to-lipid ratio was 2 g irinotecan per 10 g HSPC. The relevant formulation information was presented in Table 1 in detail.

In-vitro release studies

Irinotecan release from different vesicles was monitored using a fluorescence dequenching assay. Before analysis, a sample 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 employed. The fluorescence intensity data were collected continually with a time interval of ~8 s. The excitation/emission wavelengths were 381/420 nm, respectively. Temperature was controlled at 37°C using a jacketed sample holder, which was connected to a circulating water bath. The achieved intensity data were converted to concentration data according to a fluorescence intensity (FI) versus concentration standard curve. Percentage release was determined by $100 \times ([irino]_t - [irino]_0)/[irino]_{total}$, where $[irino]_t$ and $[irino]_0$ are free irinotecan concentrations at time points t and 0 and $[irino]_{total}$ is the total irinotecan concentration, including both free and liposomal irinotecan. To quantitatively compare the difference in drug release kinetics, percentage release was plotted as a function of time.

Animal experiments

All animal experiment protocols were approved by CSPC ZhongQi Institutional Animal Care and Use Committee (Approval number: 2009-PLI-01, 2009-PLI-02 and 2009-PLI-03; Approval date: October 12, 2009) and complied with the Regulations for the Administration of Affairs concerning Experimental Animals (Hebei Province, China).

Pharmacokinetic studies

Plasma pharmacokinetic analysis was performed in normal balb/c mice. Forty-five male balb/c mice were divided into five treatment groups with nine mice per group (three mice/sampling points, three sampling points). Each group received a single intravenous bolus dose of liposomal or free irinotecan via the tail vein (25 mg/kg). The irinotecan solution (2.5 mg irinotecan/ml) was prepared by dissolving irinotecan hydrochloride in 5% glucose and the pH was adjusted to ~4 to prevent the formation of the inactive carboxylate form. At 1 h, 4 h and 24 h, blood samples were obtained via cardiac puncture under anaesthesia and 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 additional analysis.

Irinotecan concentrations in plasma were determined using a well-established HPLC method.[12,32] For 20 µl plasma, 460 µl ice-cooled 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 Shimadzu HPLC system controlled by LC solution software was used for chromatographic analysis, which was composed of DGU-20A5 degasser, LC-20AT liquid chromatograph, SIL-20A auto-sampler, RF-10AXL detector and CTO-20A column oven. The auto-sampler and the column compartment were maintained at 25°C. The HPLC separations were achieved using a Diamonsil C18 column (150 mm × 4 mm i.d., 5 µm particle size) from Dikma (Lake Forest, USA) with a run time of 15 min at a flow rate of 1 ml/min. A guard column (Diamonisl C18, 4 mm × 8 mm) was installed ahead

Table 1 Formulation information for liposomal irinotecan

Formulation	Anion	Drug-to-lipid ratio	Mean size (nm) (PDI)	Zeta potential (−mV)	Loading efficiency (%)	Application
pLI-cd-2.9-2	sbe-CD	0.2	97.8 (0.058)	17.6 ± 1.5	99.22 ± 1.30	Release, PK and efficacy
pLI-cd-2.9-3	sbe-CD	0.3	97.6(0.054)	18.1 ± 2.1	99.19 ± 0.94	Release, PK and efficacy
pLI-cd-2.9-4	sbe-CD	0.4	97.2 (0.061)	16.9 ± 1.9	99.19 ± 1.80	Release, PK, efficacy and Toxicity
pLI-s-2.9-3	sulfate	0.3	97.8 (0.047)	17.3 ± 1.7	96.30 ± 2.13	Release and PK

cd, sulfobutyl ether cyclodextrin; PDI, polydispersion index. pLI, pegylated liposomal irinotecan; s, sulfate. Irinotecan was loaded into HSPC/Chol/DSPE-PEG vesicles with entrapped triethylammonium salts of sulfobutyl ether beta cyclodextrin or sulfate. In all cases, the intraliposomal concentration of triethylammonium cation was ~500 mM. PEG grafting density referred to the molar percent of DSPE-PEG relative to HSPC and in all the formulations peglipids were grafted at a density of 2.9 mol%.

of the analytical column. The excitation and emission wavelength was 381 nm and 420 nm, respectively.

The mobile phase was a mixture of acetonitrile (A) and aqueous phase (B) (3% TEA solution adjusted to pH 5.5 with acetic acid (23 : 77, v/v)). Under the HPLC conditions outlined above, the carboxylate species of irinotecan were eluted at 3 min and the lactone species eluted at 7 min.

Standard curves for the two species of the drug were generated by dissolving the drug in either 40% methanol–60% 10 mM citrate buffer (pH 3) for the lactone species or 40% methanol–60% 10 mM borate buffer (pH 9) for the carboxylate species. The recovery of both species was >95% and the standard curve with an r-value of 0.999.

Anti-tumour efficacy study

RM-1 prostate tumour cells were injected subcutaneously (5×10^5 cells/mouse) in the right flank region of male BDF1 mice. Tumours were allowed to grow to a mean tumour volume of ~0.4 cm^3 before the initiation of treatment. Tumour-bearing mice were randomly divided into five groups ($n = 8$). Liposomal or free irinotecan (25 mg/kg) was administered to the mice. Control mice were treated with an isotonic sucrose-histidine solution. The tumour size was measured using vernier caliper. Tumour volume (V) was calculated according to the equation $(\pi/6) \times \text{width}^2 \times \text{length}$. Animal weight and tumour sizes were monitored every two or three days.

Acute toxicity evaluation

The maximum tolerated dose of different irinotecan formulations following intravenous administration was evaluated in healthy female c57 mice. Briefly, the drug was administered via the tail vein in groups of two mice, beginning with a dose of 25 mg/kg irinotecan and continuing with a dose escalation factor of ~1.33 until a dose level of 78 mg/kg was achieved (dose levels: 25, 33, 44, 59.0 and 78 mg/kg). If during the observation period there was no mortality, irreversible morbidity or severe body weight loss (consistent loss in excess of 20% of original weight maintained for 72 h), the highest administered dose was considered as the acute single injection maximum tolerated dose.

In all cases, qualified animal care technicians monitored the mice for weight loss and other signs of stress/toxicity for a period of 21 days. Because death cannot be used as an end point, mice were sacrificed at the first sign of distress for humane consideration. After 21 days, all remaining mice were sacrificed and necropsies were conducted to identify any additional drug toxicity.

Statistical analysis

The results in all figures and tables are shown as the mean ± standard deviation (SD). In Table 2 the effect of the formulation on % carboxylate of the total plasma irinotecan was examined using the Kruskal–Wallis test. Post-hoc comparison of the means of individual treatments was performed using Nemenyi's test. Similarly, the data at each sampling point in Figures 2, 3 and 5 were statistically examined using the Kruskal–Wallis test. Individual differences between the various formulations were then examined using Nemenyi's test. The data in Figure 4 was analysed using the Mann–Whitney *U*-test. In all cases, $P < 0.05$ was considered to be statistically significant.

Table 2 Percentage carboxylate of total plasma irinotecan after injection of free or liposomal irinotecan

Formulation	Carboxylate (% of total)		
	1 h	4 h	24 h
pLI-cd-2.9-2	2.29 ± 0.39	2.62 ± 0.32	7.90 ± 1.49
pLI-cd-2.9-3	1.72 ± 0.37	3.57 ± 0.55	9.02 ± 2.40
pLI-cd-2.9-4	2.47 ± 0.06	2.85 ± 0.60	8.97 ± 2.27
pLI-s-2.9-3	1.50 ± 0.02	2.56 ± 0.49	27.39 ± 2.74

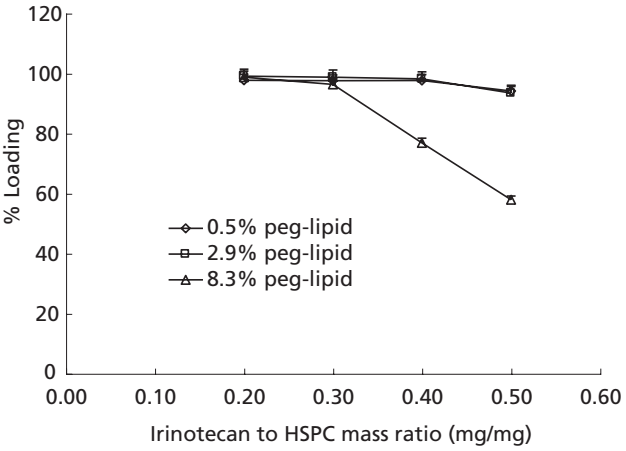

Figure 2 Drug loading profiles of irinotecan into HSPC/Chol/DSPE-PEG vesicles in response to triethylammonium salt of sbe-CD gradients. Data points represent the mean of three independent experiments and error bar represents standard deviation.

Results

Drug loading experiments

Previous work has shown that irinotecan could be loaded into vesicles in response to a transmembrane pH gradient.[17–26,28–30] In this study, to create a pH gradient, the TEA salt of sulfobutyl ether cyclodextrin was employed. In this method, the dried lipid membrane was hydrated with TEA/sbe-CD solution to form TEA/sbe-CD-containing liposomes. Following extrusion to reduce vesicle size, the liposomes were suspended in neutral sucrose–histidine buffer and thus a transmembrane TEA/sbe-CD gradient was generated. Because TEA is a weak base (pKa = 10.75), TEA dissociates to produce proton and neutral TEA, which can freely permeate the lipid bilayer. Therefore, the presence of a TEA/sbe-CD gradient means the presence of a neutral TEA gradient, which could induce the movement of neutral TEA towards extraliposomal medium. The transmembrane transportation of neutral TEA resulted in the acidification of intraliposomal media, which drives the intraliposomal accumulation of neutral irinotecan molecules.

The effects of drug-to-lipid ratio (D/L) and PEG grafting density on drug loading were investigated (Figure 2). It was found that at low and medium PEG grafting density (0.5% and

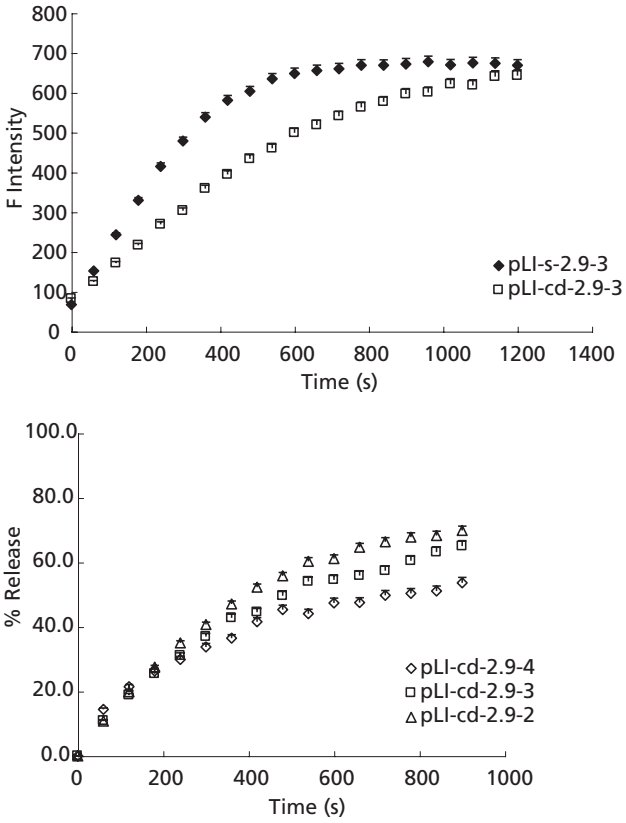

Figure 3 Effect of anions (a) and PEG grafting density (b) on drug release rate. Data points represent the mean values calculated from six repeated measures. For comparison, the fluorescence intensity in (b) was converted to % release since the three formulations had different D/L ratios.

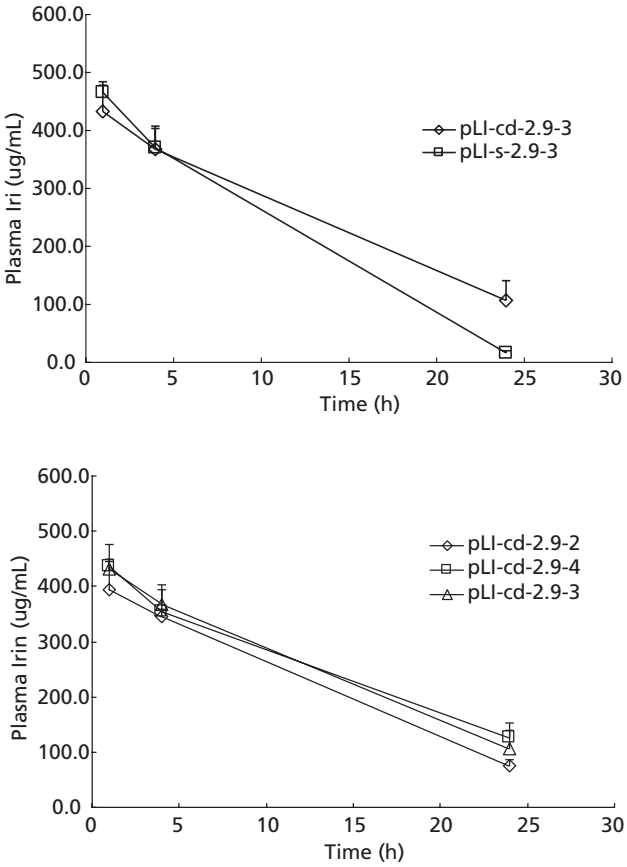

Figure 4 Plasma pharmacokinetics of liposomal irinotecan formulations in normal balb/c mice. All liposomal irinotecans were administered to balb/c mice at 25 mg/kg via tail vein ($n = 3$). Data points represent the mean irinotecan concentration ± SD. The plasma concentration of irinotecan was plotted as a function of time and regression analysis was performed. (a) sbe-CD vesicles versus sulfate vesicles. (for pLI-cd-2.9-3, $y = 463.4e^{-0.0615t}$, $t_{1/2} = 11.3$ h; for pLI-s-2.9-3, $y = 601.6e^{-0.1526t}$, $t_{1/2} = 4.5$ h). (b) vesicles different D/L ratio. (for pLI-cd-2.9-2, $y = 442.0e^{-0.0740t}$, $t_{1/2} = 9.4$ h; for pLI-cd-2.9-3, $y = 463.4e^{-0.0615t}$, $t_{1/2} = 11.3$ h; for pLI-cd-2.9-4, $y = 449.6e^{-0.0528t}$, $t_{1/2} = 13.1$ h). In all cases, r^2 values are >0.995 and not given.

2.9%), drug loading efficiency decreased slightly with increasing D/L ratio and PEG content had almost no influence. In contrast, at high PEG grafting density, the percentage loading dramatically decreased with increasing D/L ratio. It is not clear why this phenomenon occurred but the interaction of TEA with negatively charged peglipids might play a role since if the ammonium salt of sbe-CD was employed, the phenomenon could not be observed. Due to the incomplete PEG coverage of vesicles at low PEG grafting density and inefficient drug loading at high PEG density, vesicles with a PEG grafting density of ~2.9% were chosen for further investigation.

Drug release rate

To investigate drug-release profiles of different formulations, drug-release experiments were performed in NH_3-containing release media. Free NH_3 could freely permeate lipid bilayer and elevate intraliposomal pH, thus inducing drug release. Based on data presented in Figure 3a, the drug release rates for different formulations were different. The replacement of sulfate by sbe-CD could considerably improve the irinotecan retention ($P < 0.05$), indicative of the enhanced interaction of irinotecan with sbe-CD inside vesicles.

Furthermore, the D/L ratio also played a role in drug-release kinetics (Figure 3b). Vesicles with a high D/L ratio displayed relatively slow drug release under current release conditions ($P < 0.05$). Despite in-vitro release experiments not precisely mimicking the in-vivo environment, these data were still meaningful for the prediction of in-vivo release kinetics.

Plasma pharmacokinetics

Intravenous injection of free irinotecan resulted in the rapid clearance of irinotecan from plasma. Only the concentration at 1 h could be determined, with a value of 2.12 ± 1.21 μg/ml. The concentrations at 4 h and 24 h were lower than the quantification limit and could not be determined. In contrast, all liposome formulations had a prolonged circulation time of irinotecan (Figure 4). Consistent with drug-release experiments, in circulation, sbe-CD was still more able to stabilize entrapped irinotecan than sulfate and the resulting $t^1/_2$ values were 11.3 h and 4.5 h, respectively (pLI-cd-2.9-3 vs pLI-s-

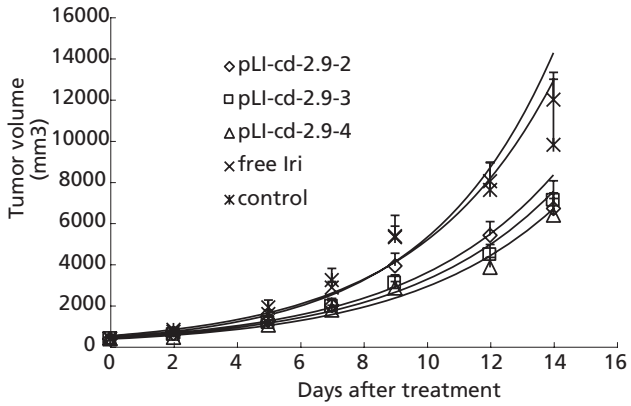

Figure 5 The antineoplastic effect of liposomal and free irinotecan in RM-1 tumour model. When the mean tumour volume reached to ~0.4 cm^3, irinotecan formulations were injected into BDF1 mice via tail vein at a dose of 25 mg/kg ($n = 8$). To reveal the exponential growth of tumour, the tumour volume was plotted as a function of time, and the exponential trend lines were also added.

2.9-3). As revealed by Mann–Whitney *U*-test, the difference between these two formulations was statistically significant ($P < 0.05$).

Since at plasma pH value (7.35–7.45), the lactone ring is liable to hydrolysis, leaked irinotecan will rapidly convert to its open ring form. Therefore, increased drug release rate might lead to decreased lactone/carboxylate ratio. Based on our results, at 24 h, percentage lactone values in the pLI-s-2.9-3 group were significantly lower than those in the pLI-cd-2.9-3 group ($P < 0.05$), indicating the rapid drug-release rate from the former formulation (Table 2).

Moreover, it was found that the D/L ratio also played a role in the irinotecan circulation time. An elevated D/L ratio led to increased irinotecan half-life, indicative of the reduced release rate at a higher D/L ratio when TEA/sbe-CD loading technology was employed.

Anti-tumour studies

The growth of RM-1 tumour in BDF1 mice was slow compared with other allograft tumour models (e.g. S-180 in KM mice). The tumour reached a volume of ~400 mm^3 two weeks after inoculation, and then liposomal and free irinotecan were administered to mice at a dose of 25 mg/kg. Statistic analysis was performed to compare the initial tumour volumes (day 0) among different groups and there was no difference ($P > 0.05$). Free irinotecan exhibited almost no therapeutic effect relative to control over all the experimental period. However, from day 2 (post first administration) to day 14, the tumour volumes in liposomal groups were significantly smaller than those of the free irinotecan and control groups ($P < 0.05$, $n = 8$). It seems that therapeutic effects of liposomal irinotecan formulations increased with elevated D/L ratio and vesicles with a high D/L ratio (pLI-cd-2.9-4) was the most efficacious (Figure 5). Regression analysis revealed that in all mice (control or treated), the tumour growth was governed by exponential equation ($V_t = V_0 e^{kt}$, wherein, V is tumour volume, t is time and k is growth constant.). The calculated tumour doubling time in mice treated with pLI-cd-2.9-4 was 3.26 days, about 1.3 fold that in the free irinotecan group (Table 3).

Table 3 The exponential growth of RM-1 tumour in male BDF1 mice

Treatment group	r^2	*P*	V_0	k	Doubling time (days)
Control	0.974	0.000	474.9	0.2524	2.75
Free	0.987	0.000	417.2	0.2627	2.64
pLI-cd-2.9-2	0.986	0.000	402.1	0.2295	3.02
pLI-cd-2.9-3	0.989	0.000	388.4	0.2155	3.22
pLI-cd-2.9-4	0.976	0.000	353.5	0.2129	3.26

RM-1 cells were inoculated into BDF1 with 5×10^5 cells/mouse. 14 days post inoculation, liposomal and free irinotecan were administered to BDF1 mice via tail vein at a dose of 25 mg/kg (8 mice /group). The mean tumour volume values were used to calculate the kinetic parameters ($V = V_0 \times e^{kt}$). The doubling time was calculated with LN(2)/k. Because individual mice were required to generate each data point and subsequent sampling over time was not possible, the standard deviations for V_0, k and doubling time could not be calculated.

Acute toxicity

Acute toxicity studies were performed using healthy c57 mice to evaluate the maximum tolerated doses of both free and liposomal irinotecan formulations. Since only the use of a small amount of mice was approved, only the formulation pLI-2.9-4 was selected. This formulation had the highest drug-to-lipid mass ratio and thus could be administered at a high irinotecan dose level. Moreover, the formulation had a slow drug-release rate and enhanced efficacy, worthy of further evaluation. However, even at the highest administered dose of 78 mg/kg, the maximum tolerated dose of liposomal irinotecan (pLI-cd-2.9-4) was not achieved (maximum body weight loss of ~6%). In contrast, at the same dose level, one mouse in the free irinotecan group exhibited > 15% body weight loss lasting for ~48 h. These data revealed that liposomal irinotecan formulation might be less toxic than free irinotecan. The result was promising since liposome-entrapped camptothecins were typically more toxic than free drugs based on previous observations. Perhaps in our studies the slow drug-release rate from vesicles counteracted the toxic effects produced by liposome encapsulation (e.g. more drugs delivered to healthy tissues in active form and prolonged drug exposure time, which might induce increased toxicity of camptothecins).

Discussion

Liposome encapsulation can alter the pharmacokinetics and biodistribution of entrapped drugs, resulting in improved safety and toxicity profiles.[31,33] However, to realize this, liposomes must be able to retain a drug in the circulation and subsequently release it at the desired site.[34,35] Premature drug release will lead to the loss of drugs in normal tissues, thus inducing severe toxicity. In contrast, slow drug release will cause reduced efficacy if the leaked drugs are not enough to inhibit tumour growth. Therefore, researchers must find an

optimum equilibrium point between drug retention and release so that the formulation with improved therapeutic index could be obtained.

Drug release rate is affected by many factors (e.g. vesicle size, lipid bilayer composition and intraliposomal medium composition).[34,35] Compared with other factors, intraliposomal medium composition may play an important role in the control of drug release, especially when drugs are encapsulated using 'active loading' technologies.[14–16] In these kind of methods, weakly basic drugs actively accumulate into vesicles in response to a pH gradient. Inside vesicles, they might convert to the protonated form, which might be precipitated by counter anions. The physical status of drug/anions directly determines drug release rate from vesicles.

However, unlike doxorubicin, it is hard to retain irinotecan inside vesicles even when a traditional 'active loading' technique is employed. To improve irinotecan retention inside vesicles, a series of methods have been tried, of which the most successful are those reported by Bally, Ramsay[17–19,22] and Kirpotin groups.[27,32] In the two former cases, the anion employed was still sulfate, but because ionophore-mediated loading technology was used, certain kinds of cations (e.g. Mg^{2+} and Cu^{2+}) were introduced into the intraliposomal media, thus resulting in relatively slow drug release. According to their studies, the $CuSO_4$ plus A23187 formulation mediated a half-life of irinotecan release of 44.4 h; a ~4-fold increase compared with other liposome formulations, indicating the importance of the divalent metal ions.[17–19,22] In contrast, other researchers tried replacing sulfate with other polyanions (e.g. sucrose octasulfate).[27,32]

In this study, we developed a modified drug-loading technology to increase the retention of irinotecan, which involved the use of sulfobutylether-β-cyclodextrin (sbe-CD) as anions in conjunction with transmembrane triethylammonium (TEA) gradient. Sbe-CD is one of the most popular beta-CD derivatives used as a pharmaceutical excipient due to an improved toxicity profile and ability to solubilize poorly water-soluble drugs.[36,37]

Since sbe-CD is a polyanionic beta-cyclodextrin derivative and has a lipophilic cavity that may accommodate the lipophilic part of drugs, it is expected that sbe-CD might interact with irinotecan via both electrostatic and hydrophobic interactions. Are these two effects sufficient to drive the accumulation of irinotecan into vesicles? To test this, a drug-loading experiment was performed using vesicles with entrapped sodium salt of sbe-CD. However, only ~5% irinotecan was loaded into vesicles. In contrast, in the presence of nigericin, ~96% loading efficiency could be achieved (D/L = 0.2, Na^+ ~300 mM). Nigericin is an ionophore that could exchange a proton from external medium with a Na^+ from intraliposomal media,[38,39] thus creating a pH gradient. Accordingly, to effectively load irinotecan into vesicles, a transmembrane pH gradient is still indispensable.

Since nigericin is hard to remove from vesicles and the residual nigericin might affect drug release (especially in plasma), we adopted a 'self-generating' system to set up the pH gradient. The TEA salt of sbe-CD was prepared by the treatment of sbe-CD Na with ion exchange chromatography, followed by titration with TEA. When a transmembrane TEA/sbe-CD gradient was created by buffer exchange, TEA could move across lipid membrane due to the high transmembrane TEA concentration gradient, thus inducing the acidification of intraliposomal media and the generation of pH gradient, which is similar to the case where an ammonium sulfate gradient is employed (Figure 6).

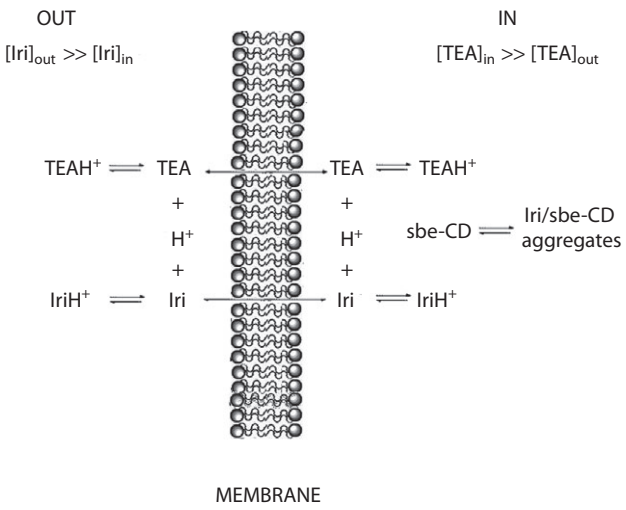

Figure 6 Schematic representation of irinotecan loading into large unilamellar vesicles in response to triethylammonium salt of sulfobutyl ether cyclodextrin gradient.

Despite the concentration gradient of sbe-CD Na not being the driving force for irinotecan loading, sbe-CD anions could markedly improve irinotecan retention relative to sulfate. The difference in drug retention might be associated with the different physical status of irinotecan/anion aggregates inside vesicles. Sbe-CD used in this study has a mean substitution degree of ~7, namely, 7 of the total 21 hydroxyl groups per CD are substituted by sulfobutyl ether groups. Because of the very low pKa value of the sulfonic acid groups, sbe-CD carries multiple negative charges at intraliposomal pH (~5–6). As for irinotecan, its pKa is 8.1; inside vesicle almost all the irinotecan molecules are in charged form (carrying a positive charge). Accordingly, each sbe-CD could bind multiple irinotecan molecules via an electrostatic effect.

Aiyama *et al.*[35,40]have proved that if the concentration of irinotecan is > 2 mM, irinotecan could self-aggregate as a dimer. In this study, the drug-to-lipid mass ratio ranged from 0.2 to 0.4. For ~100-nm vesicles, the trapped volume was assumed as 2.0–2.5 μl/umol lipid. Quick calculation revealed that the molar concentration of irinotecan inside vesicles was >100 mM. Accordingly, inside vesicles, sbe-CD and irinotecan could form complicated aggregation status. One sbe-CD molecule interacts with multiple irinotecan molecules and irinotecan from different sbe-CD/irinotecan electrostatic complexes could self-aggregate into dimers.

In contrast, one sulfate could only bind two irinotecan molecules. No physical status like sbe-CD/irinotecan aggregates could be formed. Complicated aggregation status might be responsible for the slow release rate of irinotecan from sbe-CD vesicles.

More interestingly, it was found that increased D/L ratio led to improved drug retention. This phenomenon might be

used as an evidence to prove the existence of insoluble aggregate inside vesicles. To prove this, let us first assume all the irinotecan molecules inside vesicles are soluble and drug release is governed by Fick's law. Theoretical deduction could result in the following equations:[33,35]

$$\text{Release}\ (\%) = 100 \times (1 - e^{-Kt}) \quad (1)$$

$$K = pA_m k / ([H^+]_i V_i) = 3pk / (r[H^+]_i) \quad (2)$$

Wherein, K is release constant, p is permeability parameter, A_m is membrane area, k is the dissociation constant of irinotecan, r is vesicle size and $[H^+]_i$ is the interior proton concentration.

Based on this formula, if all the intraliposomal irinotecan molecules are in the soluble form, the percentage drug release rate should be independent of the initial intraliposomal drug concentration (which is related to initial D/L ratio). However, this hypothesis is not in agreement with our observation. Therefore, sbe-CD/irinotecan must mainly exist as insoluble precipitate, which is in equilibrium with a small amount of soluble form. Under this condition, drug release could be described by the following equation[33,35]

$$d[D]_i^{tot} / dt = -pA_m([D]_i - [D]_o) / V_i \quad (3)$$

Wherein, $[D]_i^{tot}$ is the total concentration of irinotecan inside vesicles, p is permeability parameter, A_m is membrane area and $[D]_i$ and $[D]_o$ are the concentrations of irinotecan in neutral form inside or outside vesicles.

Since $[D]_i$ is >> $[D]_o$, drug release rate is positively proportional to $[D]_i$. For vesicles with a high D/L ratio, each vesicle contains more irinotecan molecules, thus resulting in large-size precipitate. According to the Ostwald–Freundlich equation, large-size precipitate is hard to dissolve due to its small specific area, so $[D]_i$ of vesicles with high D/L ratio will be smaller than that of low D/L vesicles. Accordingly, if the dissolution of precipitate is the rate-limiting step, high D/L will lead to slow drug release.

Despite increasing D/L ratio resulting in decreased drug release rate, its influence was not as great as we expected. Previous study revealed that when doxorubicin was loaded into vesicles in response to ammonium sulfate or pH gradient, minor change in D/L ratio could markedly affect drug release rate.[14,16,35] How to expound this? The chemo-physical properties of entrapped drugs must be taken into account. Unlike irinotecan, doxorubicin is liable to self-stack into fibres with decreased solubility and thus simple anions (such as sulfate) could effectively crosslink it to form precipitate with very low solubility. In contrast, irinotecan has limited self-aggregation ability and could only self-aggregate into dimer at high concentration, so precipitates with very low solubility could not be formed even when polyanionic sbe-CD was employed. Perhaps, just because of the above reason, the D/L ratio played a relatively weak role in our study.

Based on the above discussion, the self-aggregation ability of drugs is important. Only when drugs effectively self-aggregate, can insoluble precipitate be easily formed and drug release rate be significantly affected by the D/L ratio. Provided that drugs are unable to self-aggregate, even when polyanions are employed, it is also impossible to form precipitate at high D/L ratio. In all, the self-aggregation ability of the drugs and the crosslinking ability of anions co-determine drug retention inside vesicles. For drugs that have limited self-aggregation ability, anions that possess strong crosslinking ability will be needed.

Conclusions

In this study, a novel sbe-CD gradient method was developed. Using this method, irinotecan could be effectively loaded into pegylated large unilamellar vesicles and the drug retention was considerably improved. The resulting slow-release formulation exhibited prolonged irinotecan half-life and enhanced anti-tumour efficacy. Moreover, the safety was not impaired. The improvement of formulation properties and biological activity might be associated with the formation of complicated irinotecan/sbe-CD aggregates inside vesicles.

Declarations

Conflict of interest

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

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