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In-vitro cytotoxicity, in-vivo biodistribution and anti-tumour effect of PEGylated liposomal topotecan

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Abstract

In attempt to increase the accumulation of topotecan in tumours and improve its anti-cancer activity, PEGylated liposome (H-PEG) containing topotecan was prepared. The in-vitro cytotoxicity, in-vivo biodistribution pattern and anti-tumour effect of H-PEG were studied systemically. Compared with free topotecan or conventional liposome (H-Lip), H-PEG improved the cytotoxic effect of topotecan against human ovarian carcinoma A2780 and human colon carcinoma HCT-8 cells. The IC50 value (concentration leading to 50% cell-killing) of H-PEG decreased 5 fold ($P < 0.01$) and 9 fold ($P < 0.01$) against A2780 and HCT-8 cells compared with H-Lip, respectively. The results of biodistribution studies in sarcoma S_{180} tumour-bearing mice showed that liposomal encapsulation increased the concentration of total topotecan and the ratio of lactone form in plasma. H-PEG resulted in a 70-fold and 3.7-fold increase in $AUC_{0\rightarrow 24\,h}$ compared with free topotecan and H-Lip, respectively. Moreover, H-PEG increased the accumulation of topotecan in tumours and the relative tumour uptake ratio compared with free topotecan was 5.2, and higher than that of H-Lip. The anti-cancer effect studies in murine heptocarcinoma H_{22} tumour-bearing mice showed that H-PEG improved the therapeutic efficiency of topotecan and decreased the toxicity of topotecan to a certain extent compared with H-Lip. These results indicated that PEG-modified liposome might be an efficient carrier of topotecan.

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Introduction

Topotecan is a semi-synthetic derivative of camptothecin, which is an alkaloid originally isolated from the stem wood of *Camptotheca acuminate* (Wall et al 1966). In recent years, camptothecin and its analogues have been developed as highly potent anti-cancer drugs and have received considerable attention. They exert an antineoplastic effect by inhibiting the action of topoisomerase I, which is involved in DNA replication (Hsiang & Liu 1988). The important structural requirement for successful interaction with the topoisomerase I target and anti-tumour potency in-vivo is a closed α-hydroxylactone moiety (Jaxel & Kohn 1989; Giovanella & Cheng 1991). As a derivative of camptothecin, topotecan is made water-soluble by the presence of a stable, basic side-chain at carbon 9 of the A ring (Kingsbury et al 1991). It can be administered without the severe and unpredictable side effects that are associated with camptothecin sodium and it has been approved as second-line therapy for small-cell lung cancer (SCLC) and advanced ovarian cancer by the US Food & Drug Administration (FDA). However, like other camptothecins, topotecan also undergoes a pH-dependent hydrolysis (Fassberg & Stella 1992). Under physiological conditions (i.e., at pH 7.0 or above), the lactone ring of the drug readily opens to yield an inactive carboxylate form.

To protect the active lactone ring and decrease the hydrolysis rate of camptothecin and its analogues, thus improving their anti-cancer activity, liposomes have been applied to encapsulate these drugs. Early studies demonstrated that complexes of camptothecin with lipids or liposomes can stabilize the lactone moiety and maintain its biological activity (Burke 1990; Burke et al 1993; Sugarman 1996). These findings suggest that liposomes may be an effective delivery system for these drugs. Some articles have also reported that the lactone stability and anti-tumour efficacy of

topotecan encapsulated into liposomes were improved compared with those of free topotecan. Topotecan was encapsulated in low-pH liposomes initially by Burke and colleagues and their work showed that the lactone stability of topotecan improved markedly when the drug was packaged into gel-phase disteroylphosphatidylcholine (DSPC) vesicles (Burke & Gao 1994). Subramanian & Muller (1995) also used the same method to prepare liposomal topotecan and demonstrated that it was 3- to 4-fold more effective than free topotecan in stabilizing covalent topoisomerase-I–DNA intermediates inside tumour cells. Later, to solve the drawbacks of low encapsulation efficiency and low drug-to-lipid ratios obtained by passive loading procedure, the ionophore-generated proton gradient method and ammonium sulfate gradient loading procedure have been used to entrap topotecan into liposomes, and an efficient drug loading was achieved. The stability and anti-tumour activity of topotecan were enhanced drastically after being encapsulated into liposome compared with those of free topotecan (Tardi et al 2000; Liu et al 2002).

The development of stealth, or sterically-stabilized, liposomes has made targeted liposomal therapy more feasible by reducing the uptake by the mononuclear phagocyte system (MPS) and thereby prolonging circulation time (Woodle & Lasic 1992). The best examples are liposomes containing a small fraction of poly(ethylene glycol) (PEG)-derivative phospholipids, which are known to have long-circulating characteristics after intravenous injection, and to target passively to a tumour by extravasations to the more leaky vasculature in tumour tissue (Gabizon 1995; Gabizon et al 1997). In this study, to increase the accumulation of liposomal topotecan in tumour and improve the anti-tumour activity of topotecan, PEGylated liposome containing topotecan was prepared by the ammonium sulfate gradient method, and its in-vitro cytotoxicity, biodistribution and anti-tumour activity in-vivo were studied in comparison with that of conventional liposome or free drug.

Materials and Methods

Materials

Topotecan hydrochloride was purchased from Chengdu Furunde Pharmaceutical Co. (Sichuan, China). Hydrogenated soybean phosphatidylcholine (HSPC, trademark: Epikuron 200SH) was kindly provided by Degussa (Freising, Germany). PEG (MW_{2000})-phosphatidylethanolamine (PEG-PE, trademark: lipoid PEG-PE) was purchased from Lipoid GmbH (Ludwigshafen, Germany). Cholesterol was purchased from Shenyang Medicines Company (Shenyang, China). All other chemicals were of HPLC grade or analytical grade.

Preparation of liposome

Topotecan hydrochloride was encapsulated into liposome by the ammonium sulfate loading procedure (Haran 1993). Briefly, HSPC, cholesterol and PEG_{2000}-PE (1:1:0.1, mol/mol/mol) were co-dissolved in chloroform and evaporated to dryness under reduced pressure. The thin film obtained was hydrated with ammonium sulfate solution (200 mM), and the suspension was frozen and thawed 5 times, then the liposomes were extruded under high-pressure nitrogen through a sterile polycarbonate membrane (Nucleopore) 5 times (0.8, 0.45, 0.22, 0.1 μm pore size, in order) using high-pressure extrusion equipment (Lipex extrusion). After extrusion, the extra-liposomal salt was removed by dialysing against Hepes buffer (10 mM Hepes, 145 mM NaCl, pH 7.4) solutions for 24 h. Subsequently, topotecan hydrochloride in powder form was added immediately and incubated at 55 °C for 5 min with agitation and PEGylated liposome containing topotecan was obtained. As the control, conventional liposomal topotecan was also prepared in the same way without the addition of PEG_{2000}-PE.

The topotecan-containing liposomes were characterized by encapsulation efficiency and particle size. The unencapsulated topotecan was separated from liposome by a Sephadex G-50 column, and the content of drug encapsulated into liposomes was determined, then the encapsulation efficiency was calculated. The mean size of vesicle was determined using a Laser Diffraction Particle Size Analyzer (LS 230; Beckman Coulter, Inc.). All measurements were carried out at 25 °C.

In-vitro cytotoxicity assay

The cytotoxicities of PEGylated liposomal topotecan (H-PEG), conventional liposomal topotecan (H-Lip) and free topotecan were determined by methyl-thiazol-tetrazolium salt (MTT) assay as described previously (Mosmann 1983). Briefly, human ovarian carcinoma (A2780) and human colon carcinoma (HCT-8) cells were cultured in RPMI 1640 medium with 5% fetal bovine serum (FBS) and seeded on a 96-well plate (5000 cells per well). After 24 h incubation at 37°C in a humidified atmosphere of 95% air–5% CO_2, the culture medium in each well was carefully replaced with 100 μL of medium containing serial dilutions of liposomal or free topotecan samples (in triplicates). As a control, the same dose of empty liposomes was added to the culture medium. After a further 48 h incubation, 10 μL of 5 mg mL^{-1} MTT dissolved in phosphate-buffered saline (PBS) was added to each well, and the cells were incubated for another 4 h at 37 °C. The medium was then removed carefully and 150 μL of dimethyl sulfoxide (DMSO) was added to dissolve the precipitate. The value of OD at 570 nm in each well was then determined by automated plate reader.

Biodistribution study in tumour-bearing mice

S_{180} solid tumour cells were used and all tumour models were established by injecting harvested tumour cells in a single subcutaneous injection in the right axillary region of the male ICR mice (18–22 g) for the biodistribution study. The mice were purchased from the Animal Center of Shenyang Pharmaceutical University, and the study

protocol was approved by the Institutional Animal Care and Use Committee, Shenyang Pharmaceutical University, China. The tumours grew to approximately 2000 mm^3 in size after one week of implantation. The mice were then sorted according to body weight, with three mice per cage. Free topotecan solution, H-Lip and H-PEG were administered to the tumour-bearing mice by bolus intravenous injection into the lateral tail vein at a dose of 10 $mg\,kg^{-1}$ topotecan. At each time point, 0.083, 0.5, 1, 3, 6, 12 and 24 h after injection, mice were sacrificed. Blood samples (0.3 mL) were collected and centrifuged at 10 000 rev min^{-1} for 3 min and plasma (100 μL) was collected in new tubes. Different organs and tissues, such as heart, liver, spleen, lung, kidney, brain, marrow and tumour, were removed, weighed and homogenized.

Total topotecan and the lactone and carboxylate forms of topotecan were determined using fluorescence spectroscopy as described in previous studies (Warner & Burke 1997). Briefly, 300 μL ice-cold methanol was added to 100 μL plasma or other tissue-homogenate solutions to precipitate plasma proteins and solubilize the liposomes. The sample was vortexed for 10 s and centrifuged at 10 000 rev min^{-1} for 3 min. The methanolic solution was stored at −30 °C until analysis and just before HPLC analysis the sample was diluted with an equal volume of refrigerated water. The total topotecan was quantified by diluting the methanolic solution with an equal volume of buffer (pH 3.0).

Anti-cancer therapy study

Male ICR mice (18–22 g) were purchased from the Animal Center of Shenyang Pharmaceutical University. The study protocol was approved by the Institutional Animal Care and Use Committee, Shenyang Pharmaceutical University, China. The murine heptocarcinoma cells (H_{22}, 4×10^6/0.2 mL) were transplanted onto the subcutaneous tissue of the right axillary region of the mice on day 0. The mice were randomly sorted according to body weight, into four groups, with ten mice per group. The tumour-bearing mice were treated with free topotecan, H-Lip or H-PEG at a dose of 10 $mg\,kg^{-1}$ on multiple dosing on days 1, 5 and 9 through the tail vein. Mice in the control groups received injections of normal saline. Mice were weighed on every day, and the tumour size of each mouse was measured by caliper and calculated by the formula: ½($A \times B^2$), where A is length and B is width (in mm). On day 11 after tumour implantation, mice were sacrificed by cervical dislocation and the tumours were removed and weighed.

HPLC analysis

HPLC analysis used an ODS column (150 × 3.9 mm) with a run time of 15 min at a flow rate of 1.0 $mL\,min^{-1}$. The mobile phase system was TEAA buffer–acetonitrile–tetrahydrofuran (85:15:0.1, v/v) and TEAA buffer was obtained by adjusting 3% triethylamine in water to pH 5.5 with glacial acetic acid. The mobile phases were filtered and degassed before use. The fluorescence spectrofluorometric detector (excitation wavelength 380 nm, emission wavelength 525 nm) was used.

Statistical analysis

Mean and standard deviations (s.d.) were calculated using Microsoft Excel (Microsoft Office Excel 2003; Microsoft, Redmond, WA). OriginPro7.0 was used to calculate the area under the concentration–time curve from time zero to time t (AUC_{0-t}) value of topotecan in various tissues. In Figures 1 and 2, the effect of the various formulations on cell viability at each concentration or the effect of the various formulations on topotecan concentration at each time point was analysed using the analysis of variance. In

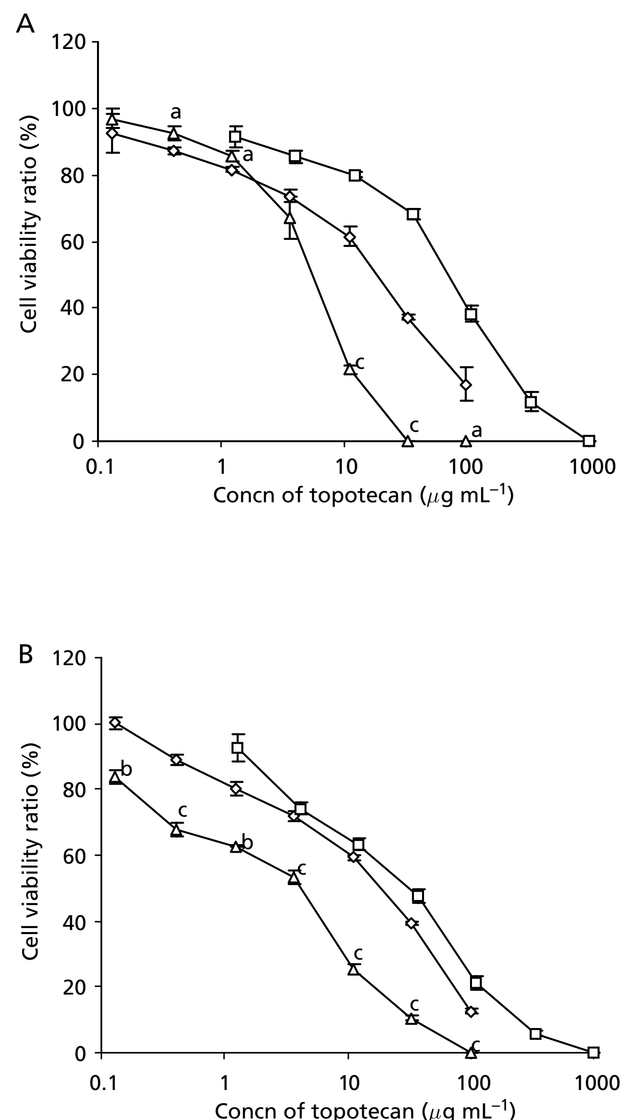

Figure 1 In-vitro cytotoxicity of H-PEG (△), H-Lip (◇) and free topotecan (□) in cultured tumour human ovarian carcinoma cells A2780 (A) and human colon carcinoma cells HCT-8 (B). Each value represents the mean ± s.d., n = 3. [a]$P < 0.05$, [b]$P < 0.01$, [c]$P < 0.001$, H-PEG vs H-Lip.

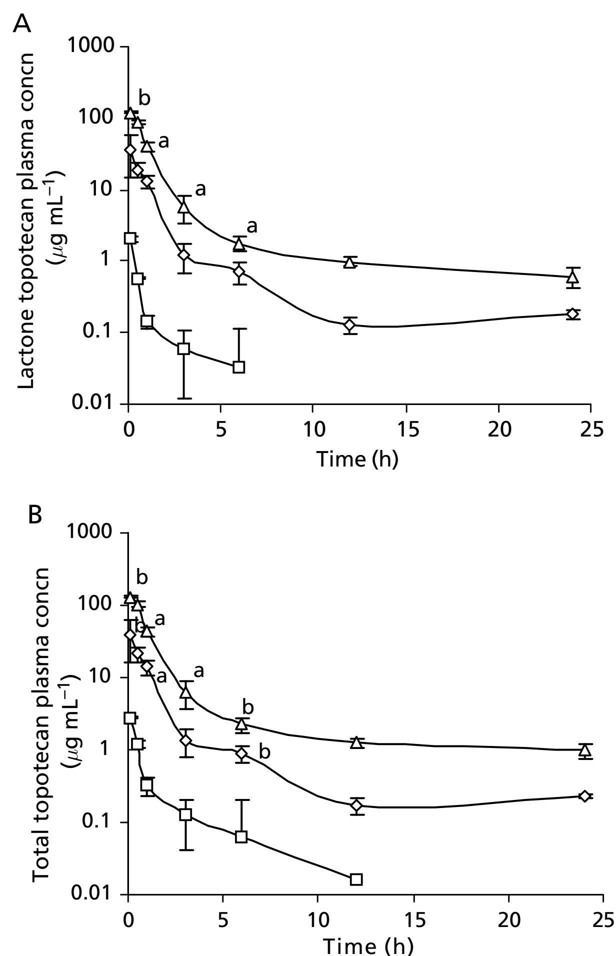

Figure 2 Concentration–time curve of topotecan in plasma based on lactone topotecan (A) or total topotecan (B) measurements after a single intravenous dose (10 mg kg^{-1}) of H-PEG (△), H-Lip (◇) or free topotecan (□) in mice. Each value represents the mean ± s.d. of 3 mice. $^{a}P < 0.05$, $^{b}P < 0.01$, H-Lip or H-PEG vs free topotecan.

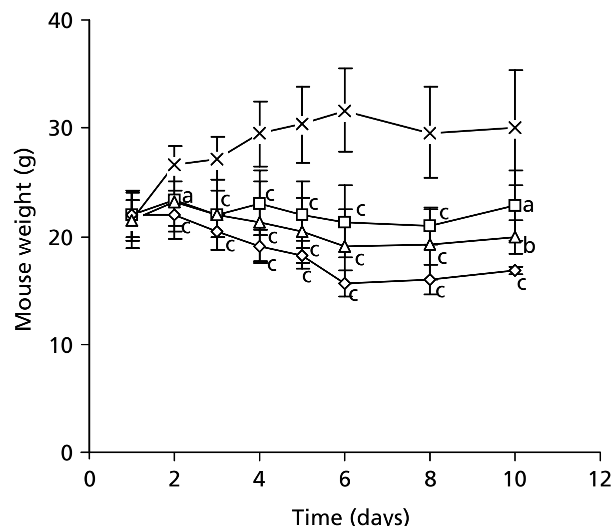

Figure 3 Weight curve of H_{22}-bearing mice treated with H-PEG (△), H-Lip (◇) or free topotecan (□). Control group received the same volume of normal saline (×). Each point represents the mean ± s.d. of 10 mice. $^{a}P < 0.05$, $^{b}P < 0.01$, $^{c}P < 0.001$, vs control group.

both cases, post-hoc comparison of the means of individual groups was performed using Dunn's test. In Figure 3, the effect of formulation type and time on the weight of mice was statistically analysed using a repeated measures analysis of variance. Individual differences between the formulations were evaluated using Tukey's test. In Tables 1 and 2, the effect of the various formulations on disposition in each tissue type or the effect of the various formulations on the various biological measurements was analysed using the analysis of variance. Individual differences between the formulations were then evaluated using Dunn's test. In all cases, statistically significant differences were assumed to be $P < 0.05$.

Results and Discussion

Liposome characterization

Liposomal topotecan was characterized by encapsulation efficiency and particle size. Liposomes containing topotecan with a high loading efficiency of about 90% and a high drug–lipid ratio of 1:9 were obtained. The encapsulation efficiency of H-Lip and H-PEG was 91.0 ± 1.3% and 90.0 ± 1.2%, respectively. Addition of PEG_{2000}-PE decreased appreciably the loading efficiency of liposome, but there was no significant difference ($P > 0.05$, Tukey's test). All liposomes extruded through 100-nm pore size filter were about 140 nm in diameter – the addition of PEG_{2000}-PE had no marked effect on vesicle size ($P > 0.05$, Tukey's test). There was also no difference in vesicle size before and after loading topotecan.

In-vitro cytotoxic effect

The cytotoxic effect of free topotecan, conventional liposomal topotecan and PEGylated liposomal topotecan against human ovarian carcinoma (A2780) and human colon carcinoma (HCT-8) cells were studied. Topotecan concentrations leading to 50% cell-killing (IC50) were determined from concentration-dependent cell viability curves. Liposomal encapsulation enhanced the antiproliferation ability of topotecan (Figure 1). The IC50 values for H-Lip and H-PEG on A2780 were 13.60 ± 3.00 $\mu g\,mL^{-1}$ and 2.54 ± 0.92 $\mu g\,mL^{-1}$, respectively, and the cytotoxicity increased about 2 fold ($P < 0.01$) and 10 fold ($P < 0.001$) compared with free topotecan (IC50 = 25.45 ± 1.69 $\mu g\,mL^{-1}$). On HCT-8 cells, H-Lip slightly increased the cytotoxicity of topotecan (IC50 = 12.90 ± 0.91 $\mu g\,mL^{-1}$) compared with free topotecan (IC50 = 16.72 ± 2.45 $\mu g\,mL^{-1}$), but there was no marked difference between them ($P > 0.05$). However, the cytotoxicity of H-PEG (IC50 = 1.43 ± 0.10 $\mu g\,mL^{-1}$) increased 12 fold ($P < 0.05$) compared with that of free topotecan. As controls, empty conventional liposomes or

Table 1 Distribution of topotecan in various tissues calculated on total topotecan after a single intravenous dose (10 mg kg^{-1}) of free and liposomal topotecan in mouse S_{180} solid tumour cell model

Group	AUC$_{0\to24h}$ of topotecan in various tissues ($\mu g\,g^{-1}h$)							
	Heart	Liver	Spleen	Lung	Kidney	Brain	Bone marrow	Tumour
Free topotecan	8.03	8.49	5.18	3.75	62.54	4.57	5.37	6.09
H-Lip	20.28^{a}	91.00^{a}	712.10^{b}	35.65^{a}	37.29	4.42	17.86	25.27^{b}
H-PEG	16.22	61.41^{b}	134.09^{c}	41.80^{c}	43.15	6.84	10.49	31.58^{b}

$^{a}P < 0.05$, $^{b}P < 0.01$, $^{c}P < 0.001$, H-Lip or H-PEG vs free topotecan.

Table 2 Comparison of anti-tumour effect against H_{22}-tumour-bearing mice in-vivo treated with free or liposomal topotecan

Group	Tumour weight (g)	Mean weight inhibition ratio (%)	Tumour volume (mm^3)	Mean volume inhibition ratio (%)
Control	2.08 ± 0.77		3676 ± 836	
Free topotecan	0.91 ± 0.62^{a}	56.3	1535 ± 596^{b}	58.2
H-Lip	0.12 ± 0.07^{b}	94.2	24.8 ± 32.5^{b}	95.8
H-PEG	0.08 ± 0.02^{b}	96.1	10.5 ± 23.7^{b}	98.7

Each value represents the mean ± s.d. of 10 mice. $^{a}P < 0.05$, $^{b}P < 0.001$, free topotecan, H-Lip or H-PEG vs control group.

PEGylated liposomes showed no cytotoxicity against cultured A2780 and HCT-8 cells.

In previous reports, many studies have demonstrated that liposomes can alter the cellular uptake of drugs in-vitro, and drugs entrapped in liposomes may be delivered intracellularly by different mechanisms. The release of the drug from liposomes and the subsequent uptake of the free drug by the cells is the important mechanism (Weinstein & Leserman 1984; Garelli 1993). The factors influencing the drug leaking from liposomes, such as lipid composition, may be critical in determining drug bioavailability to cells and the magnitude of cytotoxicity. In general, liposomes show a similar or less cytotoxic activity depending on the different phase-transition temperature of phospholipids in comparison with free drug (Horowitz et al 1992; Hu et al 1995). However, the in-vitro cytotoxicity results were opposite in our study. It was found that HSPC/cholesterol liposomes and HSPC/cholesterol/PEG_{2000}-PE liposomes containing topotecan had a greater cytotoxicity on A2780 and HCT-8 cells than free topotecan. We considered that a possible reason is the effect of the chemical stability of topotecan on its cytotoxic activity. It is known that for topotecan, or other camptothecin analogues, the intact lactone ring is the important structural requirement for anti-tumour activity (Jaxel & Kohn 1989; Giovanella & Cheng 1991). These drugs are not stable and their lactone structure can hydrolyse to the inactive carboxylate form under physiological conditions. Analysis of the HPLC stability data shows that the half-life (t½) of topotecan in PBS (pH 7.4, 37°C) is 23.6 min and the final lactone percentage at equilibrium is only 15.4% (Mi et al 1995). In contrast, liposomal encapsulation and the acid intraliposomal environment formed during drug loading by ammonium sulfate can improve the stability of the active lactone of topotecan, and our study also demonstrated the results. So during the incubation period, free topotecan may hydrolyse rapidly to the inactive carboxylate form. But for topotecan liposomes, drug may be released from the liposomes tardily and the cells are exposed to a lower dose of active drug for a long time, thus resulting in an increase in topotecan cytotoxicity in-vitro. Liu et al (2002) have also reported that topotecan encapsulated into DSPC/cholesterol liposomes is much more effective than free topotecan in inhibiting C-26 and HTB-29 cell growth.

The modification of the liposome surface with PEG-lipid prolonged circulation in the blood and increased accumulation in the tumour in-vivo has been studied in recent years. However, for PEG-modified liposome, which has a fixed aqueous layer around its surface, it is possible that liposome uptake by the cells can be suppressed as compared with the plain liposome. However, it was found that PEG-modification increased the cytotoxic effect of topotecan compared with conventional unmodified liposome containing topotecan. The IC50 value of H-PEG decreased 5 fold ($P < 0.01$) and 9 fold ($P < 0.01$) against A2780 and HCT-8 cells, respectively, compared with that of H-Lip. In a previously reported study, Sadzuka & Hirota (1998) investigated the effect of PEG-modification on the uptake of doxorubicin by Ehrlich ascites carcinoma cells in-vitro. They reported that PEG-modification of the surface of the liposomes facilitated the initial rate of

liposome uptake into the tumour cells, and this facilitation was attributed to the lipo-hydrophilic property of PEG and the fixed aqueous layer around the liposomes. Later, Sadzuka's group also examined the effect of PEG chain length and anchor length on liposomal uptake into tumour cells (Sadzuka et al 2003). They suggested that liposome adsorption or uptake into the tumour cell membrane was caused by re-uptake of PEG-lipid on the liposomal membrane and induced remaining PEG-lipids on liposomal membrane. The modification of the liposome surface with PEG-lipid increased the uptake of liposomes into tumour cells by large PEG-lipids (Sadzuka et al 2003). However, their results may not be universal because only that cell line (Ehrlich ascites carcinoma cells) was studied. So, to make it clear that H-PEG provided a more cytotoxic effect than H-Lip in our study, further studies regarding the uptake of the two liposomes by tumour cells and the intracellular concentration of topotecan are underway.

In-vivo distribution in plasma

Figure 2 shows the concentration–time curve of topotecan based on lactone form or total topotecan following a single intravenous dose ($10\,mg\,kg^{-1}$) of conventional or PEGylated liposomal topotecan and free topotecan in tumour-bearing mice. Encapsulation in liposomes significantly increased the plasma topotecan concentration. The area under the curve ($AUC_{0\rightarrow 24\,h}$) of H-Lip and H-PEG were 19 fold ($P < 0.001$) and 70 fold ($P < 0.001$) that of free drug based on total topotecan measurements. The lactone-protecting effect in-vivo was also observed. Compared with the lactone ratio of 51% for free topotecan based on AUC value, the lactone ratio of topotecan for H-Lip and H-PEG increased to 92% and 91%, respectively. This may be due to the significant protection of the lactone ring of topotecan from hydrolysis in-vivo when the drug was encapsulated into liposomes. Furthermore, the acidic intraliposomal environment formed during drug loading by the ammonium sulfate gradient method provided another important advantage in increasing the lactone stability of topotecan (Liu et al 2002). These results were similar to the earlier ones of Tardi et al (2000), who quantified the relative proportions of lactone and carboxylate after the systemic injection of free and liposomal topotecan. They reported that after injection of free topotecan, the lactone-form ratio was 48% and 52% after 5 min and 15 min, respectively. In contrast, the lactone form of topotecan was protected for liposomal topotecan, and the lactone content was still 84% at 24 h after injection (Tardi et al 2000).

Meanwhile, compared with H-Lip, H-PEG dramatically increased the plasma topotecan concentration and resulted in more than a 3.7-fold increase in $AUC_{0\rightarrow 24\,h}$ calculated on total or lactone topotecan ($P < 0.01$). Many studies have reported that several factors, including vesicle size, lipid composition, cholesterol, charge and surface hydrophilicity, have important effects on the pharmacokinetic behaviour of liposomes in-vivo (Senior & Alving 1987; Allen & Hansen 1991; Allen et al 1995). Developments in membrane biophysics have provided a new approach to producing MPS-evading liposomes. The presence of hydrophilic surface groups such as PEG on the liposome surface appears to offer steric hindrance to plasma opsonins. Consequently, the liposomes are protected from recognition and destruction by MPS cells (Storm et al 1995; Torchilin 1996; Papisov 1998). When liposomes are coated with these kinds of materials, the prolonged residence time of the vesicles in the blood is relatively independent of size, lipid dose and composition (Allen & Hansen 1991).

Distribution in liver and spleen

Compared with free topotecan, H-Lip and H-PEG obviously increased the drug distribution in liver ($P < 0.05$, $P < 0.01$) and spleen ($P < 0.01$, $P < 0.001$) (Table 1). Moreover, from our results, the uptake of H-Lip by spleen was much higher than that by liver or other organs of the reticuloendothelial system (RES). One of the possible reasons is that the content of cholesterol in our liposomal formulation is high, and the ratio of lipid to cholesterol is 1:1 (mol/mol). For cholesterol-rich vesicles, hepatic sequestration is rather poor when compared with cholesterol-free and cholesterol-poor vesicles, and such cholesterol-rich vesicles tend to localize more effectively in the spleen (Patel et al 1983; Senior et al 1985). As reported previously, PEG-modified liposomes were shown to have a decreased liver and spleen (used as an approximation of the RES generally) uptake compared with conventional unmodified liposomes. Liver and spleen uptake of H-PEG was 1.5-fold and 5.3-fold less, respectively, than that of H-Lip. That is mainly because the surface modified by hydrophilic PEG could reduce the recognition of liposomes by opsonins, and thereby decrease the RES uptake of liposomes.

Distribution in tumours

A marked increase in accumulation in tumours was found for H-Lip and H-PEG (Table 1). The relative tumour uptake ratios were 4.1 and 5.2 for H-Lip and H-PEG, respectively, versus free topotecan, based on AUC values. Compared with H-Lip, the AUC value of H-PEG increased from 25.27 to 31.58 $\mu g\,g^{-1}\,h$. As reported previously, there was an inverse relationship between liposome clearance by the RES and prolonged circulation time of liposomes. In turn, there appeared to be a direct correlation between prolonged circulation time and liposome localization in tumours (Gabizon & Papahadjopoulos 1988; Wu et al 1993; Gabizon 1995; Woodle 1995; Gabizon et al 1997). However, from our results the increase was not significant, although PEG-modification increased the plasma AUC of topotecan by 3.7 fold compared with unmodified liposome.

The reason is not highly clear. The tumour model we selected in this study is a possible factor, because the biology of the tumour cells and interaction with the microenvironment may influence the fate and effect of liposo-

mal drug. The bigger tumour (about 2000 mm^3) used in our study may decrease the uptake of PEGylated liposome by the tumour. Since PEGylated liposomes are targeted to the tumour via the vasculature, tumour vascular volume and blood flow rate are likely to be the major determinants of liposomal localization. Harrington et al (2000) have examined the relationship between tumour size and uptake of ^{111}In-DATP-labelled PEGylated liposomes. An inverse correlation between tumour weight and liposome uptake was observed. For the small tumours, their high vascular volumes comprised of relatively immature leaky neovasculature may increase the level of liposome uptake. In addition, the interstitial pressure in small deposits may be lower than in large deposits, irrespective of their state of vascularization. In contrast, the low levels of liposome uptake in large tumours were likely to be due to a relatively low vascular volume, reflecting areas of poor perfusion or even necrosis, coupled to a high tumour interstitial pressure acting to limit extravasation (Harrington et al 2000).

This result may also be related to the tumour-induced increase in liposome elimination from the circulation. Some studies have reported that liposome elimination in animals bearing well-established tumours increased for animals bearing a subcutaneous S180 or Lewis lung carcinoma (LLC) (Oku et al 1992; Parr et al 1997). The loss of circulating lipid because of the presence of tumour was different between conventional liposomes and liposomes containing PEG-PE. Parr et al (1997) have also reported that DSPC/cholesterol/PEG-PE liposomes do not result in improved doxorubicin delivery to Lewis lung solid tumours as compared with DSPC/cholesterol liposomes. Also, DSPC/cholesterol liposomes gave a T_e value (a drug-targeting efficiency parameter, relating the AUC in the circulation to the tumour AUC, $T_e = AUC_T/AUC_P$) of 0.76, which was higher than that for PEG-PE containing liposome (Parr et al 1997). However, the actual reason leading to the result in our study still needs to be further proved and explained in a later study.

Distribution in lung, bone marrow and other tissues

The distribution of topotecan in lung also changed (Table 1). The topotecan concentration in lung increased markedly when the drug was encapsulated into H-Lip and H-PEG ($P < 0.05$, $P < 0.001$) compared with that of free topotecan. The AUC value for H-PEG at 24 h post-injection was 11-fold more than that of free topotecan, and slightly higher than that of H-Lip. It can be suggested that liposomal encapsulated topotecan may be favourable for therapy of lung cancer, and further study on the therapeutic effect of the liposomal topotecan on lung cancer, especially small-cell lung cancer, should be performed.

In this study, we also determined the content of topotecan in bone marrow. Myelosuppression has been proven to be a commonly encountered dose-limiting toxicity (DLT) leading to anaemia, neutropenia and thrombocytopenia for all of the administration schedules of topotecan (Creemers et al 1996a, b). The AUC values of topotecan at 24 h post-injection for different formulations are shown in Table 1. The liposomal encapsulation slightly increased the topotecan accumulation in bone marrow, but there was no significant difference between H-Lip or H-PEG and free topotecan ($P < 0.05$). Moreover, we also determined the distribution of drug in other tissues (Table 1). The kidney is the main elimination pathway of topotecan in-vivo, and the topotecan concentration in the kidney for free drug group ($AUC_{0\rightarrow 24\,h} = 62.54\ \mu g\,g^{-1}\,h$) was the highest compared with H-Lip and H-PEG in our study. The liposomal encapsulation changed the distribution of topotecan in-vivo and decreased the elimination of drug by the kidney. H-PEG slightly increased the distribution of topotecan in heart or brain compared with free drug, but there were no marked differences ($P > 0.05$).

Anti-tumour effect

To investigate the therapeutic advantage of PEG-modified liposomal topotecan, we studied the effect of H-Lip and H-PEG in the H_{22} tumour-bearing mice model. H-Lip, H-PEG and free topotecan were given at a dose of 10 $mg\,kg^{-1}$ by intravenous injection on days 1, 5 and 9. The mean tumour size reached $3676 \pm 836\ mm^3$ in the normal saline group on day 11. Compared with the control saline group, mice receiving free topotecan showed moderate growth delay (mean tumour size, $1535 \pm 596\ mm^3$; tumour inhibition ratio, 58.2%). However, a significant delay in tumour growth rate was observed in the H-Lip and H-PEG groups, and mean volume inhibition ratios were 95.8% and 98.7%, respectively. At the end of treatment, the mice were sacrificed and the tumours were removed and weighed; the mean tumour weight of mice receiving the different treatments are shown in Table 2. In accord with the volume inhibition ratio, H-PEG and H-Lip also exhibited a better anti-tumour effect than free topotecan based on the loss of tumour weight. However, there was no significant difference between H-PEG and H-Lip ($P > 0.05$), though the tumour inhibition ratio of H-PEG was higher than that of H-Lip.

During treatment, the weight of H_{22}-bearing mice in the control group increased gradually due to the growth of tumours and was higher than that of the treated groups ($P < 0.05$). In comparison with free-topotecan-treated mice, the weight of mice in the two liposome-treated groups lightened, but there was no significant difference ($P > 0.05$) (Figure 3). This indicated that liposome encapsulation does not remarkably increase the toxicity of topotecan while improving its anti-tumour effect. The decrease of mice weight in the H-PEG group was less than that in the H-Lip group, suggesting that PEG-modified liposomes containing topotecan may be more advantageous than conventional liposome.

Conclusions

PEGylated liposomes containing topotecan had improved in-vitro cytotoxic activity against human ovarian carcinoma A2780 and human colon carcinoma HCT-8 cells. The biodistribution studies in-vivo showed that liposomal encapsulation improved the drug stability in-vivo. PEGylated liposomes had prolonged circulation time and increased tumour uptake of topotecan compared with conventional liposomes or free drug. In-vivo anti-tumour effect against H_{22} tumour-bearing mice exhibited that PEGylated liposomal encapsulation could improve the therapeutic efficiency and not increase the toxicity of topotecan versus unmodified liposomes. These results indicated that PEGylated liposomes would be an effective carrier of topotecan and can exert better anti-tumour effect than free drug or conventional liposomes containing topotecan.

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