Available online at www.sciencedirect.com
ScienceDirect
International Journal of Pharmaceutics 353 (2008) 251–259
INTERNATIONAL JOURNAL OF PHARMACEUTICS
www.elsevier.com/locate/ijpharm

Pharmaceutical Nanotechnology

The effect of PEG coating on *in vitro* cytotoxicity and *in vivo* disposition of topotecan loaded liposomes in rats

S. Dadashzadeh [a,b,*], A.M. Vali [a,c], M. Rezaie [d]

[a] *Department of Pharmaceutics, School of Pharmacy, Shaheed Beheshti Medical University, Tehran, Iran*
[b] *Pharmaceutical Research Center, Shaheed Beheshti Medical University, Tehran, Iran*
[c] *Department of Pharmaceutics, School of Pharmacy, Zanjan University, Zanjan, Iran*
[d] *Department of Toxicology and Pharmacology, School of Pharmacy, Shaheed Beheshti Medical University, Tehran, Iran*

Received 17 July 2007; received in revised form 18 October 2007; accepted 10 November 2007
Available online 23 November 2007

Abstract

Amphoteric drugs encapsulated in PEGylated liposomes may not show superior therapeutic antitumor activity due to increased leakage rate of these drugs in presence of PEG-lipids. In order to investigate the effect of PEG coating on *in vitro* and *in vivo* characteristics of topotecan loaded liposomes, an amphoteric anticancer drug, PEGylated and conventional liposomes were prepared by lipid film hydration method. Various properties of the prepared nanoliposomes such as encapsulation efficiency, size, zeta potential, physical stability as well as the chemical stability of lactone form of topotecan, cytotoxicity and topotecan pharmacokinetics were evaluated. *In vitro* cytotoxic activity was evaluated on murine Lewis lung carcinoma (LLC) and human mammary adenocarcinoma (BT20) cells. Pharmacokinetic was evaluated in Wistar rats after *i.v.* injection of topotecan, formulated in PBS pH 7.4 or in conventional or in PEGylated liposomes. The conventional liposome (CL) formulation was composed of DSPC/cholesterol/DSPG (molar ratio; 7:7:3), while for PEGylated liposome the composition was DSPC/cholesterol/DSPG/DSPE-PEG_{2000} (molar ratio; 7:7:3:1.28). The size of both liposomes was around 100 nm with polydispersity index of about 0.1. In comparison with free drug, liposomal topotecan showed more stability for topotecan lactone form *in vitro*. Compared to free topotecan, PEGylated and conventional liposomes improved cytotoxic effect of topotecan against the two cancer cell line studied. The results of pharmacokinetic studies in rats showed that both CL and PEGylated liposomal formulations increased the concentration of total topotecan in plasma, however, initial concentration and the values of AUC, MRT and $t_{1/2\beta}$ were much higher ($P < 0.001$) for PEGylated liposomal drug than for conventional one or free drug. PEGylated liposome resulted in a 52-fold and 2-fold increases in $AUC_{0\text{-infinity}}$ compared with that of free topotecan and CL, respectively. These results indicated that PEG modified liposome might be an effective carrier for topotecan.

Keywords: Topotecan; Conventional liposome; PEGylated liposome; Pharmacokinetic; Cytotoxicity; Stability

1. Introduction

Topotecan, a semisynthetic derivative of camptothecin, is a potent inhibitor of DNA topoisomerase I and has demonstrated encouraging antitumor activity in a wide variety of tumors (D'Arpa and Liu, 1989). Topotecan is used for the treatment of ovarian cancer after failure of initial or subsequent chemotherapy and small-cell lung cancer after failure of first-line chemotherapy and is increasingly being combined with other standard chemotherapeutic agents for improved therapy (Abraham et al., 2004). Similar to camptothecin and other derivatives, topotecan is a cell cycle-specific drug and acts as stabilizer of complex of DNA and topoisomerase I (Kingsbury et al., 1991; Hertzberg et al., 1989; Caserini et al., 1997). Therefore, it is advantageous to expose tumor cells to the drug for a prolonged period. This point is supported by clinical observations that patients refractory to topotecan exhibited increased response rates when the drug was administered as a low-dose infusion (Hochster et al., 1994). As with all camptothecins, however, topotecan undergoes a pH dependent hydrolysis of the lactone ring to form a relatively inactive carboxylate in aqueous solutions and in plasma. Therefore, encapsulation of topotecan

* Corresponding author at: School of Pharmacy, Shaheed Beheshti University of Medical Sciences, No. 105, Shams Alley, across from Tavaneer Avenue, Vali-e-Asr Avenue, P.O. Box 14155-6153, Tehran, Iran. Tel.: +98 21 88773521; fax: +98 21 88795008.
E-mail address: Sdadashzadeh@sbmu.ac.ir (S. Dadashzadeh).

0378-5173/$ – see front matter © 2007 Elsevier B.V. All rights reserved.
doi:10.1016/j.ijpharm.2007.11.030

within liposomes seems to be a potential solution to overcome the abovementioned problems.

Liposomes have previously been used as carriers for anticancer drugs, and they have shown a significant decrease in the amount and types of nonspecific toxicities and an increase in the amount of drug that can be effectively delivered to the tumor (Papahadjopoulos, 1999; Gabizon and Martin, 1997; Martin, 1998). Liposomes can also provide slow release of an encapsulated drug, resulting in sustained exposure to tumor cells and enhanced efficacy (Tardi et al., 2000). In addition to these advantages, the properties of topotecan, i.e. S phase-specific cytotoxicity and fast inactivation at physiological pH, make it worthwhile to develop liposomal topotecan. An earlier study by Burke and Gao (1994) showed that large (500 nm diameter) multilamellar vesicles with an acidic interior could stabilize topotecan as the lactone form. Unfortunately, multilamellar vesicles have limited value as drug carriers because of their rapid clearance from the circulation (Allen and Stuart, 1999). The conventional liposomes have been made by Liu et al., suffered from rapid elimination rate of topotecan from blood circulation, as elimination half-life for liposomal topotecan was 2.9 h, which was nearly the same as for free topotecan (2.6 h). They explained this phenomenon by the possibility that topotecan does not form a gel-like precipitate with ammonium sulfate as doxorubicin does (Liu et al., 2002). Also these authors employed a clearly high dose of topotecan in their experiments (5 mg/kg versus usual recommended dose of 1.5–2.5 mg/m^2 or 0.04–0.07 mg/kg) which could reflect cytotoxicity against phagocytic cells and block RES activity that is normally responsible for rapid clearance of drug loaded carriers (Bally et al., 1990). Therefore, a slower distribution phase shown in their study for liposomal topotecan in comparison to free drug maybe a consequence of RES blockage caused by distinct high dose of topotecan. The same reason is conceivable for the conventional liposomes made by Tardi et al. (2000).

Recent studies have demonstrated that specific type of liposomes, also known as long-circulating or sterically stabilized liposomes (SLs), can circulate in the blood for prolonged periods of time without being trapped in the mononuclear phagocytic system (MPS). The best example is that of liposome formulations containing a small fraction of polyethylene glycol (PEG)-derivatized phospholipid, which has been shown to alter dramatically the pharmacokinetic properties of doxorubicin (DOX), leading to long elimination half-life and small volume of distribution (Gabizon et al., 1994; Unezaki et al., 1995; Daemen et al., 1997). Besides these efficiencies, however, it has been reported that characteristics of loaded drug could markedly influence the capacity of PEG coated liposomes in increasing the plasma residence of drug (Webb et al., 1998). Amphoteric drugs encapsulated in PEGylated liposomes may not show superior therapeutic antitumor activity due to increased leakage rate of these drugs in presence of PEG-lipids. For example vincristine plasma levels were not affected by the presence of PEG-lipid in liposome bilayer (Webb et al., 1998). Also Gabizon et al. (1996) demonstrated that some DOX-loaded anionic liposome formulations demonstrated an efficacy similar to that of SL-DOX.

Therefore, the object of the present study was to investigate the effect of PEG coating on *in vivo* behavior of topotecan loaded liposomes. To this end conventional and PEGylated liposomal formulations containing topotecan were prepared and compared in terms of cytotoxic activity and pharmacokinetic.

2. Material and methods

2.1. Chemicals

Topotecan was obtained from Ohua Pharmaceutical Technology; 1,2-Distearoyl-*sn*-Glycero-3-Phosphocholine (DSPC), 1,2-Distearoyl-*sn*-Glycero-3-[Phospho-*rac*-(1-glycerol)] (DSPG), and 1,2-Distearoyl-*sn*-Glycero-3-Phosphoethanolamine-*N*-[Carboxy(Polyethylene Glycol)2000] (DSPE-PEG$_{2000}$) was purchased from Lipoid GmbH (Switzerland). Cholesterol, sodium chloride, potassium chloride, disodium hydrogen phosphate, potassium dihydrogen phosphate and Triton X-100 were supplied from Merck (Darmstadt, Germany). Cellulose dialysis tubing (12000 MWCO) was supplied by Biogen (USA).

2.2. Liposome preparation and characterization

Topotecan was encapsulated in the liposomes, either conventional or PEGylated. Different liposome formulations composed of the bilayer forming phospholipid, DSPC, in combination with different amount of cholesterol and DSPG and contained 0 and 7 mol% of DSPE-PEG$_{2000}$ (Table 1) were prepared by the thin film hydration technique (Hope et al., 1985) and reported in detail elsewhere (Vali et al., in press). Briefly, lipids were dissolved and mixed in chloroform:methanol (5:1). After removing the organic solvents, dried lipid films were hydrated with two different concentrations of topotecan in PBS pH 5 (drug to lipid molar ratios, 1:30 and 1:60) at 65 °C to achieve a final lipid concentration of 10 mg/ml. Following hydration, the multilamellar vesicles (MLVs) were extruded 10 times through stacked polycarbonate filters with 0.1 μm pore size at 60 °C using a water-jacketed Extruder^{TM} (Northern lipids, Vancouver BC, Canada). Unencapsulated topotecan was removed from the preparation by dialysis at 4 °C against 100 volumes of phosphate buffer solution (PBS) pH 7.4 for 24 h.

Size distribution of liposomes was monitored by photon correlation spectroscopy using a Coulter Model N4SD submicron particle analyzer (Coulter Electronics, FL, USA). Zeta potentials of liposomes were determined using 90 PLUS particle size analyzer with ZETA PALS system, Brookhaven Corp. (Hostville, NY) at 25 °C.

The amount of topotecan incorporated in liposomes was determined by HPLC method developed in our lab (Vali et al., 2005). The liposomal suspension was disrupted by adding 0.1 ml acidic methanol (1% perchloric acid in methanol) and 10 μl of TritonX-100 20% to 0.1 ml of drug loaded liposomes. Twenty microlitre of the solution was injected into a Novapack C_{18} column. The drug was eluted with 0.05 M ammonium acetate, acetonitrile and triethylamine (84:16:1.50, v/v) containing tetrabutyl ammonium hydrogen sulfate (2 mM) adjusted to

Table 1
Characteristics of liposome containing topotecan

Phospholipid composition	Drug to lipid molar ratio[a]	Encapsulation efficiency (%)	Topotecan concentration (μg/ml)[b]	Stability (4 °C)
A DSPC/CHOL/DSPG 9:4.5:1	1:60	9.96 ± 1.19	11.42	1 day
B DSPC/CHOL/DSPG/DSPE-PEG_{2000} 7:3.5:3:1.02	1:60	7.27 ± 1.28	6.64	3 days
C DSPC/CHOL/DSPG 7:3.5:3	1:30	6.70 ± 1.26	14.87	1 day
D DSPC/CHOL/DSPG/DSPE-PEG_{2000} 9:4.5:1:1.09	1:30	2.36 ± 0.58	4.43	3 days
E DSPC/CHOL/DSPG 9:9:1	1:30	3.76 ± 1.01	9.57	<1 day
F DSPC/CHOL/DSPG 7:7:3	1:60	7.61 ± 1.79	9.28	<1 day
G DSPC/CHOL/DSPG/DSPE-PEG_{2000} 9:9:1:1.43	1:60	0.68 ± 0.12	0.70	3 day
H DSPC/CHOL/DSPG/DSPE-PEG_{2000} 7:7:3:1.28	1:30	11.44 ± 1.12	22.50	3 day
I DSPC/CHOL/DSPG 7:7:3	1:30	6.21 ± 0.98	15.15	1 day

[a] Ratio between mol drug and mol lipid used in the preparation of liposomes.
[b] Drug concentration after separating unentrapped drug from liposome preparations.

pH 5 with hydrochloric acid. Detection was performed fluorimetrically with an excitation wavelength of 380 nm and an emission wavelength of 527 nm. The amount of drug entrapment in liposomes was calculated using the following equation:

$$\%\ \text{Drug entrapment efficiency} = \frac{\text{Amount of topotecan in liposomes}}{\text{Total amount of topotecan used in liposome preparation}} \times 100$$

Here, the amount of initially added drug was regarded as that of total drug, because the drug loss in the preparation process was negligible (data not shown).

Conventional and PEGylated liposomes containing topotecan were evaluated for physical stability in the storage condition (PBS buffer pH 7.4) at 4 °C and in human plasma incubated at 37 °C. The physical stability of liposomal topotecan formulations kept at 4 °C were evaluated by monitoring drug leakage for 1 week by removing portions of liposomes from a pool stored at 4 °C at various time points for 1 week. Changes in mean diameter were also monitored.

To determine the effect of liposomal formulation on the stability of topotecan lactone form, the lactone ring opening rate for free topotecan and optimized liposomal topotecan formulations as a result of hydrolysis were also evaluated (Vali et al., in press).

2.3. Cell culture

Murine Lewis lung carcinoma (LLC) and human mammary adenocarcinoma (BT-20) were purchased from the American Type Culture Collection (Manassas, VA). LLC cells were maintained in Dolbecco's modified eagle's medium (DMEM) cell culture medium supplemented with FBS to 10%, Napyruvate to 1 mM, and penicillin and streptomycin to 50 units/ml and 50 μg/ml, respectively. BT-20 cells were maintained in Earl's minimal essential medium (EMEM) cell culture medium supplemented with FBS to 10%, Na-pyruvate to 1 mM, non-essential amino acids and L-glutamine to 1 mM each.

2.4. Cytotoxicity assay

The cytotoxicity of optimized preparations of the liposomal topotecan against LLC and BT20 cells was studied using a MTS test. A ready-for-use CellTiter 96® Aqueous One solution of MTS (Promega, Madison, WI) was used according to a protocol suggested by the manufacturer. Formulations with topotecan concentration of up to 2.5 μg/ml dispersed in Hank's buffer were

added to cells grown in 96-well plates to about 75% confluence, in three replicates. After 24 h of incubation at 37 °C, 5% CO_2 plates were washed three times with Hank's buffer followed by the addition of 20 μl of CellTiter 96® Aqueous One solution. After 1 h incubation at 37 °C, 5% CO_2, the cell survival rate was estimated by measuring the color intensity of the MTS degradation product at 492 nm using an ELISA plate reader. Assay was performed twice for each cell line. Full concentration dependences have been obtained for conventional and PEGylated loaded topotecan samples. Additional control with topotecan-free liposomes was done at the fixed concentration of 10 mg/ml, since no difference from the "no treatment" sample or any concentration dependence was expected in this case. Corresponding IC_{50} values were determined from the cytotoxicity data using Prism software.

2.5. Animals and surgical procedures

Eighteen male Wistar rats from Pasteur Institute (Tehran, Iran) weighing 255 ± 42 g (mean ± SD) were used and divided into three groups ($n = 6$, in each group). All animals were acclimatized in wire cages in a 12 h light–dark cycle for a minimum of 5 days before the beginning of the experiment to allow them to adjust to the new environment. During this period, they had free access to food and water.

The day before the experiment, the rats were anesthetized with *i.p.* injection of a ketamine/xylazine solution (80 mg/kg ketamine, 12 mg/kg xylazine). An anesthetized and surgically prepared animal was positioned under a dissecting scope in dorsal recumbence. A 2 cm ventral cervical skin incision was made right of the midline with its caudal terminus at the level of the clavicle to expose the right pectoral muscle. Underlying salivary and lymphatic tissues were separated by means of blunt dissection to visualize the right common jugular vein. Five millimeters of vessel cranial to the site where the jugular vein passes under the clavicle was mobilized. A sterile polyethylene 20 (PE20) cannula was inserted into the vessel and secured in place with suture. A 0.5 cm midline skin incision was made between the scapulae. Hemostats were used to draw the PE20 by the port back through the scapular incision. Skin incisions were closed and the cannula was flushed by heparinized physiological saline to prevent clogging of the cannula. The cannula port was sealed with a sterile stainless steel pin. 23 gauge blunted needle.

After surgery, rats were housed individually in cages and allowed to acclimate for 1 day before pharmacokinetic analysis. Rats were fed with rat chow and water and maintained on a 12 h light–dark cycle. The jugular vein cannula was kept patent by flushing with heparinized physiological saline every 24 h.

2.6. Drug administration and sample collection

The animals were treated with, optimized PEGylated and conventional liposomes containing 2.5 mg/m^2 of topotecan. For comparison, a pharmacokinetic evaluation of free drug was performed. Topotecan was dissolved in PBS pH 7.4 rights before the *i.v.* injection. The different formulations were injected via jugular vein. Serial blood samples (0.1 ml) were collected from individual rats through the jugular vein cannula using a 1 ml syringe. After each blood sample, cannula was flushed with 20 μl of heparinized saline. Plasma was separated by centrifugation, mixed with 0.1 ml of cold methanol and stored at −20 °C until analysis. A typical blood sampling schedule after *i.v.* dosing was 0, 0.25, 0.5, 1, 2, 4, 6, 8, and 10 h and variously thereafter up to 48 h, depending on the formulation.

2.7. Pharmacokinetic analysis

Pharmacokinetic analysis was performed by two compartmental open model using following exponential equation (Shargel et al., 2005):

$$C_t = A \times e^{-\alpha t} + B \times e^{-\beta t}$$

where C_t is the drug concentration (Y-axis) at time t (X-axis). A and B are the Y-intercepts, and α and β are the apparent first order distribution and elimination rate constants. Elimination rate constant (β) was estimated by least square regression of plasma concentration–time data points lying in the terminal log-linear region of the curve. Rate constant for distribution phase (α) was obtained by the method of residuals. The area under the plasma concentration-versus-time curve (AUC) was calculated using the trapezoidal rule with extrapolation to infinity. Clearance (Cl) was calculated by dividing dose over AUC. Volume of distribution at steady state (V_{ss}) and mean residence time (MRT) were calculated using following noncompartmental equations:

$$V_{ss} = \text{Dose} \times \frac{\text{AUMC}}{(\text{AUC})^2}$$

$$\text{MRT} = \frac{\text{AUMC}}{\text{AUC}}$$

where AUMC (area under the first moment curve) is the area under the $C \times t$ plotted against t from time 0 to infinity (Shargel et al., 2005).

2.8. Statistical analysis

One way ANOVA with Tukey post test were used for statistical analysis to determine significant differences between group means. Statistical significance was established at $P < 0.05$.

3. Results and discussion

The observation that long-circulating liposomes of small size (<100 nm) accumulated in the interstitial fluid of transplanted tumors at levels comparable to those in RES-rich organs, such as liver (Gabizon, 1992; Huang et al., 1992), was the basis for a renewed momentum in the search of liposomal drug formulations with potential applications in cancer therapy. When anthracyclines were encapsulated in long-circulating liposomes, a superior therapeutic index was demonstrated in various experimental animal tumor models (Papahadjopoulos et al., 1991; Gabizon, 1992). One of the factors with major impact on the

circulation time of liposomes is the inclusion of a small fraction of PEG-derivatized phospholipids. The resulting coating of the liposome surface with PEG is expected to increase surface hydrophilicity, decrease opsonization and RES uptake, and prolong liposome circulation time (Woodle and Lasic, 1992). However, lack of distinct advantages, from the pharmacokinetics and/or efficacy points of view, in the presence of PEG coating has also been reported (Webb et al., 1998; Gabizon et al., 1996). It seems that characteristics of loaded drug can markedly influence the capacity of PEG coated liposomes in increasing the plasma residence of drug. In this regard the effect of PEG coating on *in vitro* characteristics and *in vivo* behavior of topotecan loaded liposomes was investigated in the present study.

3.1. Preparation and characterization of topotecan liposomes

Topotecan liposome formulations, either conventional or PEGylated, were prepared by extruding multilamellar liposomes using different cholesterol and DSPG ratios in phospholipid mixtures (Table 1). Hydration of the drug-lipid film, followed by 10 cycles of extrusion through 0.1 μm polycarbonate filters, was found to be a feasible preparation method for homogenous small unilamellar vesicles, with mean diameter in range of 95–103 nm with a polydispersity values of around 0.1. The liposomal compositions which were used for further *in vitro* and *in vivo* evaluations, were optimized to obtain maximal stability and topotecan incorporation (Vali et al., in press).

As shown in Table 1, PEGylated and conventional liposomes using 3.34 mol% of topotecan, composed of DSPC/CHOL/DSPG/DSPE-PEG (molar ratio; 7:7:3:1.28) (formulation H) and DSPC/CHOL/DSPG (molar ratio; 7:7:3) (formulation I), had higher concentration of topotecan (22.50 and 15.15 μg/ml, respectively) and therefore were selected for further characterization, cytotoxicity and pharmacokinetic studies.

The optimized PEGylated (formulation H) liposome formulation had less negative charge in comparison of related conventional liposome formulation (formulation I) (mean zeta potential value of −10 and −22 mV, respectively). This could be attributed to the masking of some of the anionic charges of DSPG by DSPE-PEG_{2000}.

3.2. Stability

3.2.1. Physical stability of liposomes

PEGylated and conventional liposomes containing topotecan were physically stable for about 3 days and 1 day at 4 °C, respectively, and retained at least 95% of their initial drug content over that period. During storage no appreciable variation ($P > 0.05$) of liposome size was detected by photon correlation microscopy and no drug precipitation or liposome aggregation was observed.

The release profiles of topotecan from optimized PEGylated liposomes (formulation H) and conventional liposomes (formulation I) in PBS pH 7.4 and human plasma was also evaluated. In the employed media about 50 and 68% of drug was released over a period of 10 h from PEGylated and conventional liposomes, respectively. In both cases the release profiles were followed by a prolonged release up to more than 48 h (Vali et al., in press). The followed delayed release may be attributed to diffusion of the dissolved drug within the core of the liposome into the dissolution media. The relatively prolonged topotecan release from PEGylated liposome (formulation H) in comparison of related conventional liposome (formulation I) may be attributed to the bilayer rigidity (Arifin and Palmer, 2005; Gaber et al., 1998). In general, the more rigid the bilayer, the slower the release of drug. Thus, PEGylated liposomes containing topotecan showed a significant decrease in release rate and an increase in resistance to release, in comparison with conventional liposomes containing topotecan. The effect of human plasma on the release profile appeared to be non-significant for both liposomes).

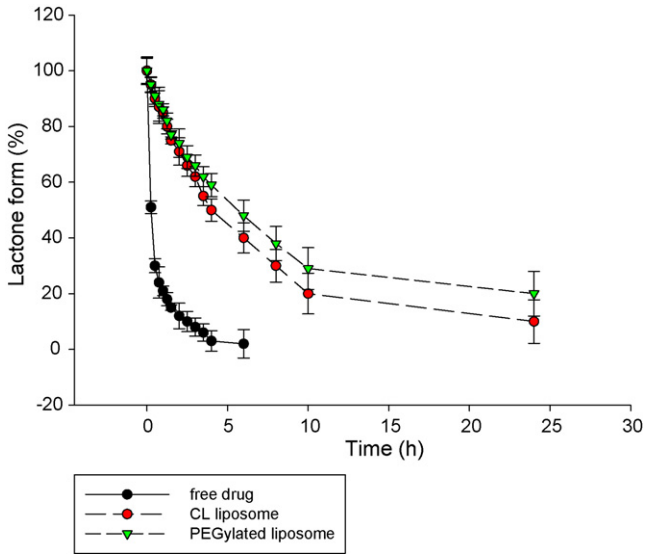

Fig. 1. Stability of lactone form of topotecan loaded in PEGylated liposomes, CL liposomes and free topotecan (control) in plasma at 37 °C.

3.2.2. Stability of lactone ring form of topotecan after loading into liposomes

Fig. 1 depicts the changes of lactone percentage at physiological pH in plasma as a function of time. Stability data were fitted on two exponential decay equation. Half-lives were determined from the two exponential decay equation using non-linear regression module of Sigmaplot 9. Hydrolysis of free topotecan proceeded quickly with a short half-life ($t_{50\%}$ value) of about 21 min. In contrast, for liposomal topotecan, the stability of the lactone moiety was markedly enhanced by liposome encapsulation. The half-lives for converting of topotecan lactone form to carboxylate form as PEGylated and conventional liposome preparations were 5.58 and 4.31 h, respectively, and the difference were not statistically significant. After 10 h incubation of conventional liposomes and PEGylated liposomes with plasma at 37 °C, 20 and 29 percent of topotecan were remained in lactone form, respectively. In overall the results of stability evaluation showed that the lactone ring of topotecan was notably preserved upon liposome encapsulation.

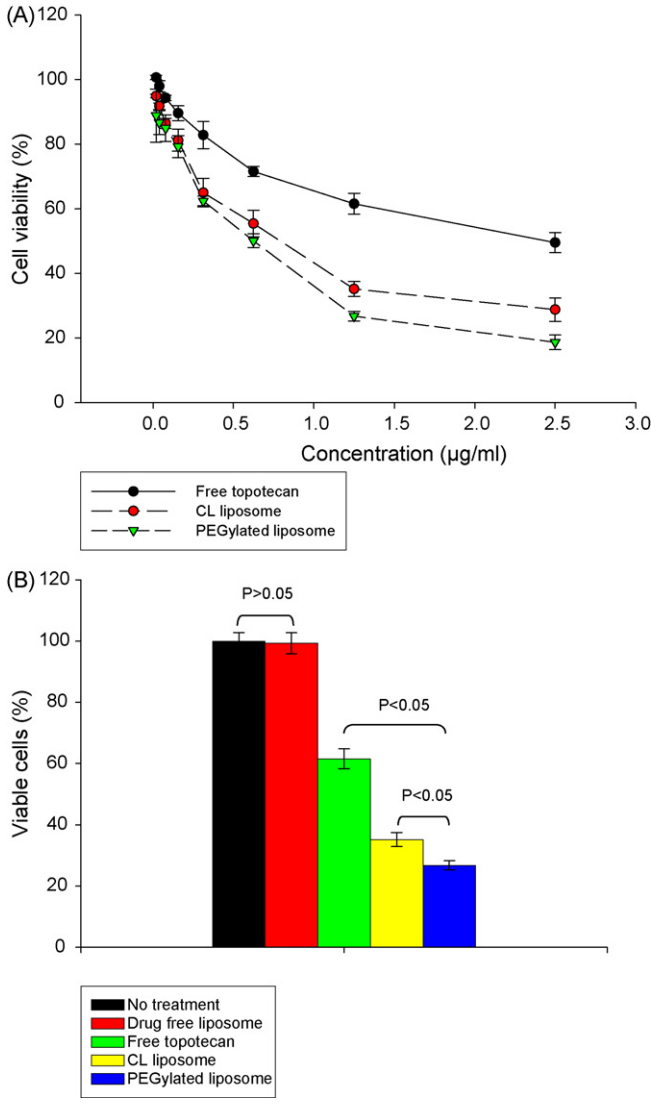

Fig. 2. Murine LLC cells. (A) Cytotoxic effect of different concentrations of PEGylated liposomes, CL liposomes and free topotecan on LLC cells, and (B) cytotoxicity of various preparations at the fixed concentration of PEGylated and CL liposome (as 1.25 μg/ml free topotecan).

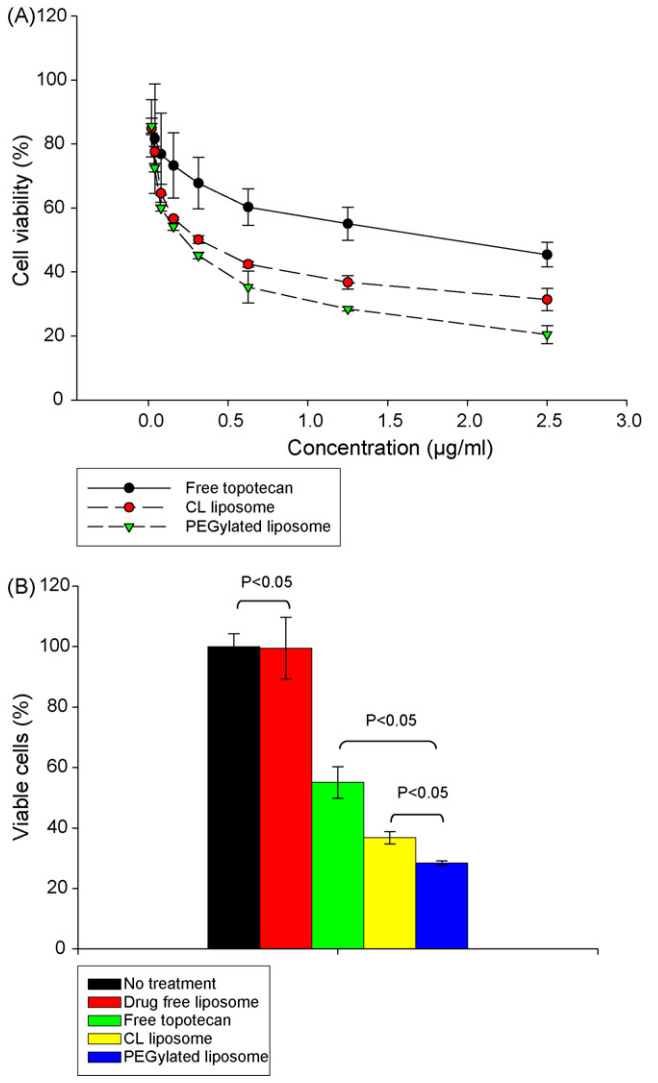

Fig. 3. Human BT20 cells. (A) Cytotoxic effect of different concentrations of PEGylated liposomes, CL liposomes and free topotecan on BT20 cells, and (B) cytotoxicity of various preparations at the fixed concentration of PEGylated and CL liposome (as 1.25 μg/ml free topotecan).

3.3. In vitro cytotoxicity assay

The cytotoxicity of different liposomal preparations (PEGylated liposomes loaded with topotecan, CL liposomes containing topotecan, free topotecan at the same topotecan concentration and PEGylated and CL empty liposomes) was investigated using two cancer cell lines; murine LLC and BT20 tumor cell lines. Figs. 2 and 3 demonstrate the typical results obtained at various topotecan concentrations of free topotecan (up to 2.5 μg/ml), PEGylated liposomes loaded with topotecan, and CL liposomes containing topotecan as well as cytotoxicity "cross-section" at topotecan concentration of 1.25 μg/ml showing the lack of any cytotoxic effect of empty PEGylated and CL liposomes.

PEGylated and CL liposome formulations demonstrated the highest toxicity against the two cancer cell lines studied. After 24 hours, the PEGylated liposome formulation killed 81.32 and 79.55% of cells in the case of LLC and of BT20 cells, respectively. The best control formulation, free topotecan, killed only 50.46% of LLC cells and 50.63% of BT20 cells. The difference between PEGylated and CL liposomes and the most cytotoxic control formulation was statistically significant ($P < 0.001$) in all studied cases. Moreover, there was no significance difference in cytotoxicity between the PEGylated liposomes and CL liposomes, in both the murine LLC and BT20 cancer cell lines except the last two concentrations. The IC_{50} values for different preparations of topotecan were also determined. In LLC cell line, the IC_{50} of PEGylated liposomes was calculated as 0.63 μg/ml compared to 0.78 μg/ml in case of CL liposomes and 2.49 μg/ml for free topotecan, and in BT20 cell line the corresponding values were 0.23, 0.33, and 1.88 μg/ml, respectively. There was no significant difference between the IC_{50} values of PEGylated and CL liposomes, in both LLC and BT20 cell lines. Concisely, the PEGylated liposome formulation was 4–8-folds more toxic than the most toxic control, the free topotecan in PBS buffer.

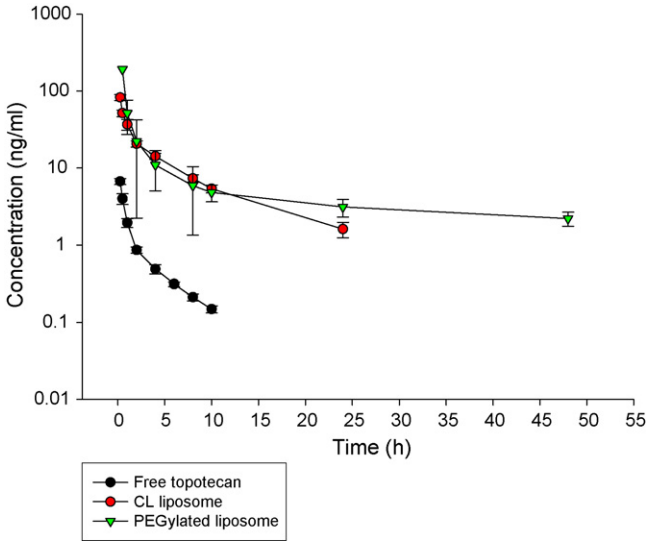

Fig. 4. Plasma concentration–time curve for topotecan following bolus intravenous injection of 2.5 mg/m^2 of free topotecan, topotecan loaded CL and PEGylated liposomes to rats. Each point represents the mean ± SD of six animals.

As we showed in the topotecan hydrolysis test, free topotecan displayed a rapid hydrolysis kinetic with a short half-life of about 21 min, whereas PEGylated and conventional liposomes encapsulated topotecan exhibited an enhanced stability with half-lives of 5.58 and 4.31 h, respectively. For the reason that the intact lactone moiety is structurally important for biological activity (Liu et al., 2002) liposomal topotecan, which could preserve the lactone moiety, was much more effective than free drug to inhibit LLC and BT20 cells growth (Figs. 2 and 3). Moreover due to the S phase-specific cytotoxicity of topotecan (Lorence and Nessler, 2004), expose of cancer cells for prolong time in contact with anticancer drug could be another reason for observed higher antitumor activity of topotecan in the case of liposomal formulations.

3.4. Pharmacokinetic study

The mean plasma concentration–time profiles of topotecan following *i.v.* bolus dosing of topotecan as free topotecan, CL liposomes, and PEGylated liposomes in rats are shown in Fig. 4.

Pharmacokinetic parameters were obtained by both two compartmental and non-compartmental analysis, as described earlier. The mean pharmacokinetic parameters for topotecan after *i.v.* administration of different compositions in rats are presented in Table 2 and the comparisons of pharmacokinetic parameters for free topotecan and topotecan loaded in conventional and PEGylated liposomes are shown in Table 3. As shown in Fig. 4 after *i.v.* bolus administration free topotecan followed a biphasic pattern with a rapid distribution phase ($t_{1/2\alpha}$ = 0.439 h) and a relatively rapid terminal elimination phase ($t_{1/2\beta}$ = 2.854 h). This result was in consistent with previous studies (Van Warmerdam et al., 1995). Similarly, plasma concentration–time profiles of topotecan for both CL and PEGylated liposomal formulations followed biphasic pattern but with a much slower elimination phase, compared with free topotecan. Encapsulation of the topotecan in liposome formulations (particularly PEGylated liposome) markedly slowed down the elimination phase and resulted in about 2.6-fold ($P < 0.001$) and 12.4-fold ($P < 0.001$) increase in the elimination half-life ($t_{1/2\beta}$) values for CL and PEGylated liposomal topotecan, respectively (Tables 2 and 3).

These results were not similar to the earlier ones of Liu et al. (2002), who studied the pharmacokinetics of topotecan conventional liposomes prepared by ammonium sulfate gradient. The liposomes made by Liu et al., suffered from rapid elimination rate of topotecan from blood circulation, as there was a little difference in $t_{1/2\beta}$ between free topotecan and liposome formulation (2.6 and 2.9 h, respectively). They explained this phenomenon by the possibility that topotecan does not form a gel-like precipitate in the presence of ammonium sulfate as doxorubicin does, therefore, has poor liposomal retention in the presence of serum.

Although Liu et al., showed a slower distribution phase and therefore a higher initial concentration value for the conventional liposome, compared to free drug, but we attribute this observations to the RES blockage by the high dose of liposomal topotecan used in their study. They administered a drug dose of 5 mg/kg to mice which is considerably higher than topotecan recommend usual dose (i.e. 1.5–2.5 mg/m^2 or 0.04–0.07 mg/kg). Drug induced inhibition of RES activity has previously been reported (Bally et al., 1990). This inhibition or "RES blockade" is believed to reflect cytotoxicity against phagocytic cells responsible for the rapid clearance of drug loaded carriers. The

Table 2
Pharmacokinetic parameters of free topotecan, CL liposomal, and PEGylated liposomal topotecan in rats after a single *i.v.* dose of 2.5 mg/m^2 (n = 6, in each group)

PK parameter	Free topotecan		CL liposome		PEGylated liposome	
	Mean	SD	Mean	SD	Mean	SD
$t_{1/2\alpha}$ (h)	0.439	0.085	0.693	0.145	0.522	0.100
$t_{1/2\beta}$ (h)	2.854	0.328	7.601	1.137	35.375	3.244
C_0 (ng/ml)	11.14	1.94	112.60	8.95	296.14	64.30
V_d (L)	20.30	2.34	1.83	0.23	4.83	1.11
V_C (L)	3.46	0.67	0.335	0.025	0.133	0.036
Cl (L/h)	4.94	0.39	0.167	0.011	0.094	0.014
MRT (h)	3.17	0.24	7.91	1.29	36.24	5.03
V_{SS} (L)	15.32	1.33	1.31	0.17	3.44	0.930
$AUC_{0\text{-inf}}$ (ng h/ml)	7.78	0.50	225.0	14.3	406.3	55.0
$AUMC_{0\text{-inf}}$ (ng h^2/ml)	24.70	2.76	1789.3	374.9	14583.3	1943.7

Table 3
Comparison of the pharmacokinetic parameters of free topotecan, conventional and PEGylated liposomal topotecan by ANOVA with Tukey post test.

Compared formulations		$t_{1/2\alpha}$ (h)	$t_{1/2\beta}$ (h)	C_0 (ng/ml)	V_c (L)	V_d (L)	Cl (L/h)	MRT (h)	V_{ss} (L)	$AUC_{0\text{-inf}}$ (ng h/ml)	$AUMC_{0\text{-inf}}$ (ng h^2/ml)
Free topotecan	CL liposome	5.49 $P<0.01$	5.83 $P<0.01$	6.63 $P<0.001$	19.78 $P<0.001$	30.15 $P<0.001$	64.16 $P<0.001$	3.87 $P<0.05$	36.29 $P<0.001$	16.22 $P<0.001$	3.78 $P<0.05$
Free topotecan	PEGylated liposome	1.80 $P>0.05$	39.95 $P<0.001$	18.62 $P<0.001$	21.06 $P<0.001$	25.26 $P<0.001$	64.91 $P<0.001$	26.97 $P<0.001$	30.78 $P<0.001$	29.75 $P<0.001$	31.20 $P<0.001$
CL liposome	PEGylated liposome	3.69 $P<0.05$	34.12 $P<0.001$	11.99 $P<0.001$	1.28 $P>0.05$	4.90 $P<0.01$	0.75 $P>0.05$	23.10 $P<0.001$	5.50 $P<0.01$	13.53 $P<0.001$	27.42 $P<0.001$

same reason is conceivable for the the PEGylated liposomes prepared by Hao et al. (2005).

Pharmacokinetic profiles of liposomal drug showed significant differences for conventional versus PEGylated topotecan liposomes. Although liposome preparations showed almost a similar distribution phase (Table 3) ($t_{1/2\alpha}=0.693$ and 0.522 h for CL and PEGylated liposomal drug, respectively) but the terminal elimination phase was 4.6 times slower for PEGylated liposomes ($t_{1/2\beta}=35.375$ h) than for conventional ones ($t_{1/2\beta}=7.601$ h). The higher value for $t_{1/2\beta}$ of PEGylated liposome formulation could be explained by the reduced MPS uptake and clearance rate of these carriers. After 24 h, PEGylated and conventional liposomes were still retained in the plasma, whereas conventional liposomes had two times less concentration in circulation. Topotecan plasma concentration following administration of PEGylated liposomes was undoubtedly higher than CL liposomes for up to 48 h. In particular, AUC increased upon encapsulation of topotecan in liposomes. The AUC was markedly higher (>28.9) for both liposomal topotecan as compared to free topotecan.

Analogously, doxorubicin was detected in the blood up to 24 h after *i.v.* injection in a study administering long-circulating liposomes to nude mice bearing human tumors (Unezaki et al., 1995; Daemen et al., 1997; Siegal et al., 1995).

Further pharmacokinetic analysis showed that, initial concentrations and the values of AUC and MRT were much higher for PEGylated liposomal drug than for conventional one or free drug (Tables 2 and 3). These results are consistent with the results of Lasic and coworkers that showed an increase in AUC of PEGylated liposomes in comparison to conventional liposomes (Lasic et al., 1991).

The extent of topotecan distribution was also reduced noticeably by liposome formulations. The volume of distribution (V_d) of the drug encapsulated in CL and PEGylated liposomes were 11.1 ($P<0.001$) and 4.2 times ($P<0.001$) less than that for free drug. Also, values of apparent volume of distribution for central compartment (V_c) and steady state volume of distribution (V_{ss}) were significantly lower for the carrier loaded drug than respective values for the free drug. It therefore appeared that encapsulation of topotecan in CL or PEGylated liposomes considerably delayed the kinetics of drug transfer from the central compartment to the peripheral tissue compartment resulted in reduced drug distribution. It should be pointed out that modulation of drug release rate by carriers has a high impact on drug distribution and in this regard topotecan release from liposomes seems was not instantaneous upon administration.

Differences between the distribution volumes (V_d and V_{ss}) of the two liposome topotecan formulations were also investigated and were found to be significant ($P<0.01$). The transcapillary passage that can contribute to the extravascular distribution of long-circulating liposomes may explain the increase of V_d and V_{ss} for PEGylated liposomes compared to conventional liposomes. The distribution of principal components of commonly used liposomes are restricted in the vascular space due to their size (>25 nm) except when blood vessels are leaky such as in inflammation tissues, tumor tissues and sinusoidal tissues such as liver, spleen and bone marrow (Harashima and Kiwada, 1996). Long-circulating liposomes according to their intrinsic characteristics, remain in blood for prolong time and this characteristic sometimes permit them to extravasate from blood compartment more than conventional liposomes.

As indicated in Tables 2 and 3, the mean clearance value of conventional liposomes and PEGylated liposomes were almost the same, but it differed significantly for liposomal formulations and free topotecan.

4. Conclusions

According to the cytotoxicity evaluation on two cancer cell lines both of topotecan loaded CL and PEGylated liposomal formulations showed markedly higher toxicity compared to free drug, however, PEG coating had no significant effect on the antitumor activity of the relevant conventional liposome against studied cell lines. The enhanced antitumor activity of liposomal topotecan can be accounted by its ability to maintain a higher portion of active lactone form for prolong time whereas free topotecan is quickly hydrolyzed into its inactive carboxylate form. *In vivo* results indicated that compared to free drug, incorporation of topotecan into the prepared liposomes distinctly improved the disposition behavior of topotecan and the degree of improvement was more noticeable for PEGylated ones. Compared to conventional liposome an approximately 2-fold increase in the topotecan $AUC_{0\text{-infinity}}$ was achieved by incorporating a PEG-lipid grafting density of 7 mol% into the bilayer. In overall, regarding topotecan, as an amphoteric drug, presence of PEG coating not only did not increase the leakage rate of drug from the optimized liposome formulation, but also was more favorable for the long-circulation and consequently better accu-

mulation in tumors than CL liposomes and particularly free topotecan.

Acknowledgements

This work was supported by a grant from Pharmaceutical Sciences Research Center, Shaheed Beheshti Medical University, IRAN. The authors acknowledge Ms. Zahra Abbasian for her valuable technical assistances.

References

Abraham, S.A., Edwards, K., Karlsson, G., Hudon, N., Mayer, L.D., Bally, M.B., 2004. An evaluation of transmembrane ion gradient-mediated encapsulation of topotecan within liposomes. J. Control. Release 96, 449–461.

Allen, T.M., Stuart, D.D., 1999. Liposome pharmacokinetics: classical, sterically stabilized, cationic liposomes and immunoliposomes. In: Janoff, A.S. (Ed.), Liposomes: Rational Design. Marcel Dekkar, New York, pp. 63–87.

Arifin, D.R., Palmer, A.F., 2005. Physical properties and stability mechanisms of poly(ethylene glycol) conjugated liposome encapsulated hemoglobin dispersions. Artif. Cells Blood Substit. Immobil. Biotechnol. 33, 137–162.

Bally, M.B., Nayar, R., Masin, D., Hope, M.J., Cullis, P.R., Mayer, L.D., 1990. Liposomes with entrapped doxorubicin exhibit extended blood residency times. Biochim. Biophys. Acta 1023, 133–139.

Burke, T.G., Gao, X., 1994. Stabilization of topotecan in low pH liposomes composed of distearoylphosphatidylcholine. J. Pharm. Sci. 83, 967–969.

Caserini, C., Pratesi, G., Tortoreto, M., 1997. Apoptosis as a determinant of tumor sensitivity to TPT in human ovarian tumors: preclinical *in vitro*/*in vivo* studies. Clin. Cancer Res. 3, 955–961.

Daemen, T., Regts, J., Meesters, M., Ten Kate, M.T., Bakker-Woudenberg, I.A.J.M., Scherphof, G.L., 1997. Toxicity of doxorubicin entrapped within long-circulating liposomes. J. Control. Release 44, 1–9.

D'Arpa, P., Liu, L.F., 1989. Topoisomerase-targeting antitumor drugs. Biochim. Biophys. Acta 989, 163–177.

Gaber, M.H., Ghannam, M.M., Ali, S.A., Khalil, W.A., 1998. Interaction of Doxorubicin with phospholipid monolayer and liposomes. Biophys. Chem. 70, 223–229.

Gabizon, A., 1992. Selective tumor localization and improved therapeutic index of anthracyclines encapsulated in long-circulating liposomes. Cancer Res. 52, 891–896.

Gabizon, A., Chemla, M., Tzemach, D., Horowitz, A.T., Goren, D., 1996. Liposome longevity and stability in circulation: effects on the *in vivo* delivery to tumors and therapeutic efficacy of encapsulated anthracyclines. J. Drug Target 3, 391–398.

Gabizon, A., Isacson, R., Libson, E., Kaufman, B., Uziely, B., Catane, R., et al., 1994. Clinical studies of liposome encapsulated doxorubicin. Acta Oncol. 33, 779–786.

Gabizon, A., Martin, F., 1997. Polyethylene glycol-coated (pegylated) liposomal doxorubicin. Rationale for use in solid tumours. Drugs 54, 15–21.

Hao, Y.L., Deng, Y.J., Chen, Y., Wang, K.Z., Hao, A.J., Zhang, Y., 2005. *In-vitro* cytotoxicity, *in-vivo* biodistribution and anti-tumour effect of PEGylated liposomal topotecan. J. Pharm. Pharmacol. 57, 1279–1287.

Harashima, H., Kiwada, H., 1996. Liposomal targeting and drug delivery: kinetic consideration. Adv. Drug Deliv. Rev. 19, 425–444.

Hertzberg, R.P., Caranfa, M.J., Holden, K.G., 1989. Modification of the hydroxy lactone ring of camptothec: inhibition of mammalian topoisomerase I and biological activity. J. Med. Chem. 32, 715–720.

Hochster, H., Liebes, L., Speyer, J., 1994. Phase I trial of low-dose continuous TPT infusion in patients with cancer: an active and well-tolerated regimen. J. Clin. Oncol. 12, 553–559.

Hope, M.J., Bally, M.B., Webb, G., Cullis, P.R., 1985. Production of large unilamellar vesicles by a rapid extrusion procedure. Characterization of size distribution, trapped volume and ability to maintain a membrane potential. Biochim. Biophys. Acta 812, 55–65.

Huang, S.K., Lee, K.D., Hong, K., Friend, D.S., Papahadjopoulos, D., 1992. Microscopic localization of sterically stabilized liposomes in colon carcinoma-bearing mice. Cancer Res. 52, 5135–5143.

Kingsbury, W.D., Boehm, J.C., Jakas, D.R., 1991. Synthesis of water-soluble (aminoalkyl)-camptothecin analogues: inhibition of topoisomerase I and antitumor activity. J. Med. Chem. 34, 98–107.

Lasic, D.D., Martin, F.J., Gabizon, A., Huang, S.K., Papahadjopoulos, D., 1991. Sterically stabilized liposomes: a hypothesis on the molecular origin of the extended circulation times. Biochim. Biophys. Acta 1070, 187–192.

Liu, J.J., Hong, R.L., Cheng, W.F., Hong, K., Chang, F.H., Tseng, Y.L., 2002. Simple and efficient liposomal encapsulation of topotecan by ammonium sulfate gradient: stability, pharmacokinetic and therapeutic evaluation. Anticancer Drugs 13, 709–717.

Lorence, A., Nessler, C.L., 2004. Camptothecin, over four decades of surprising findings. Phytochemistry 65, 2735–2749.

Martin, F.J., 1998. Clinical pharmacology and antitumor efficacy of DOXIL (pegylated liposomal doxorubicin). In: Lasic, D.D., Papahadjopoulos, D. (Eds.), Medical Applications of Liposomes. Elsevier Science, New York, pp. 635–688.

Papahadjopoulos, D., 1999. Steric stabilization an overview. In: Janoff, A.S. (Ed.), Liposomes Rational Design. Marcel Dekker, New York, pp. 1–12.

Papahadjopoulos, D., Allen, T.M., Gabizon, A., Mayhew, E., Matthay, K., Huang, S.K., et al., 1991. Sterically stabilized liposomes: improvements in pharmacokinetics and antitumor therapeutic efficacy. Proc. Natl. Acad. Sci. U.S.A. 88, 11460–11464.

Shargel, L., Wu-Pong, S., Yu, A.B.C., 2005. Applied Biopharmaceutics and Pharmacokinetics, fifth ed. Appleton & Lange, Connecticut, pp. 67–98.

Siegal, T., Horowitz, A., Gabizon, A., 1995. Doxorubicin encapsulated in sterically stabilized liposomes for the treatment of a brain tumor model: biodistribution and therapeutic efficacy. J. Neurosurg. 83, 1029–1037.

Tardi, P., Choice, E., Masin, D., Redelmeier, T., Bally, M.B., Madden, T.D., 2000. Liposomal encapsulation of topotecan enhances anticancer efficacy in murine and human xenograft models. Cancer Res. 60, 3389–3393.

Unezaki, S., Maruyama, K., Ishida, O., Suginaka, A., Hosoda, J., Iwatsuru, M., 1995. Enhanced tumor targeting and improved antitumor activity of doxorubicin by long-circulating liposomes containing amphipatic poly(ethylene glycol). Int. J. Pharm. 126, 41–48.

Vali, A.M., Shafaghi, B., Dadashzadeh, S., 2005. Simple and sensitive high performance liquid chromatographic method for the simultaneous quantitation of the lactone and carboxylate forms of topotecan in human plasma. J. Chromatogr. B Anal. Technol. Biomed. Life Sci. 818, 205–212.

Vali, A.M., Toliyat, T., Shafaghi B., Dadashzadeh, S. Preparation, optimization and characterization of topotecan loaded PEGylated liposomes using factorial design. Drug Dev. Ind. Pharm., in press.

Van Warmerdam, L.J., Ten Bokkel Huinink, W.W., Rodenhuis, S., Koier, I., Davies, B.E., Rosing, H., et al., 1995. Phase I clinical and pharmacokinetic study of topotecan administered by a 24-hour continuous infusion. J. Clin. Oncol. 13, 1768–1776.

Webb, M.S., Saxon, D., Wong, F.M., Lim, H.J., Wang, Z., Bally, M.B., et al., 1998. Comparison of different hydrophobic anchors conjugated to poly(ethylene glycol): effects on the pharmacokinetics of liposomal vincristine. Biochim. Biophys. Acta 1372, 272–282.

Woodle, M.C., Lasic, D.D., 1992. Sterically stabilized liposomes. Biochim. Biophys. Acta 1113, 171–199.