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Vascular targeting of doxorubicin using cationic liposomes

Jun Wu [a], Alice Lee [a,b], Yanhui Lu [a], Robert J. Lee [a,b,c,*]

[a] *Division of Pharmaceutics, College of Pharmacy, The Ohio State University, Columbus, OH 43210, USA*
[b] *NSF Nanoscale Science and Engineering Center (NSEC) for Affordable Nanoengineering of Polymeric Biomedical Devices (CANPBD), The Ohio State University, Columbus, OH 43210, USA*
[c] *NCI Comprehensive Cancer Center (CCC), The Ohio State University, Columbus, OH 43210, USA*

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Abstract

Tumor vessel has been recognized as an important target for anticancer therapy. Cationic liposomes have been shown to selectively target tumor endothelial cells, thus can potentially be used as a carrier for chemotherapy agents. In this study, cationic liposomes containing 20 mol% cationic lipid dimethyl dioctadecyl ammonium bromide (DDAB) and loaded with doxorubicin (DOX) were prepared and characterized. The cationic liposomal DOX showed 10.8 and 9.1 times greater cytotoxicity than control PEGylated liposomal DOX in KB oral carcinoma and L1210 murine lymphocytic leukemia cells, and 7.7- and 6.8-fold greater cytotoxicity compared to control neutral non-PEGylated liposomal DOX, repectively, in these two cell lines. Although cationic liposomal DOX had higher tumor accumulation at 30 min after intravenous administration compared to control liposomes ($p < 0.05$), DOX uptake of these liposomes at 24 h post-injection was similar to that of PEGylated liposomal DOX ($p > 0.05$) and approximately twice the levels of the free drug and non-PEGylated liposomes. In a murine tumor model generated using L1210 cells, increased survival rate was obtained with cationic liposomal DOX treatment compared to free DOX ($p < 0.01$), neutral liposome control ($p < 0.01$), as well as PEGylated liposomes ($p < 0.05$). In conclusion, the cationic liposomal DOX formulation produced superior in vitro cytotoxicity and in vivo antitumor activity, and warrants further investigation.

Keywords: Cationic liposomes; Vascular targeting; Doxorubicin; Cancer

1. Introduction

Physiological barriers have been shown to hinder effective delivery of drugs to tumors (Jain, 1998; Pluen et al., 2001). In order to reach cancer cells, a therapeutic agent must first cross the vasculature and then travel through the interstitium. Vascular targeting avoids these barriers by attacking the blood supply instead of cancer cells, either suppressing vessel formation (antiangiogenic therapy) or abolishing established vascular networks (antivascular therapy) (Denekamp, 1982; Thurston et al., 1998). Subsequently, tumor cells undergo apoptosis as a consequence of impaired nutrient and oxygen supply. Due to their high rate of proliferation, tumor endothelial cells have increased susceptibility to cytotoxic drugs (Denekamp, 1999). Furthermore, these cells are more genetically stable and are thus less prone to drug resistance compared to tumor cells. Selective delivery of cytotoxic drugs to tumor endothelium is, therefore, a promising therapeutic strategy.

Liposomes have been evaluated as a carrier of anticancer chemotherapeutic agents, such as doxorubicin (Mayer et al., 1989). Neutral liposomes exhibit preferential localization in solid tumors based on enhance permeation and retention (EPR) effect (Papahadjopoulos et al., 1991; Huang et al., 1992; Yuan et al., 1994), which relies on gradual passive accumulation of liposomes in the tumor (Huang et al., 1992; Yuan et al., 1994; Wu et al., 1993). In contrast, cationic liposomes are rapidly cleared from circulation by liver, spleen, and lung, and primarily have been used in gene delivery (McLean et al., 1997; Mahato et al., 1995; Litzinger et al., 1996). The unique properties of cationic liposomes for targeting tumor endothelium suggest that they may have utility as drug carriers. Strieth et al. (2004) and Schmitt-Sody et al. (2003) reported that paclitaxel encapsulated in cationic liposomes exhibited improved antitumor efficacy of, which was associated with impaired function of tumor microvas-

* Corresponding author at: 542 LM Parks Hall, 500 W. 12th Avenue, College of Pharmacy, The Ohio State University, Columbus, OH 43210, USA.
Tel.: +1 614 292 4172; fax: +1 614 292 7766.
E-mail address: lee.1339@osu.edu (R.J. Lee).

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culature. The study of Thurston et al. (1998) showed that uptake of cationic liposomes by endothelial cells in angiogenic blood vessels was considerably greater than that of extravasation from these vessels. Furthermore, this uptake was selective to cationic liposomes and that anionic, neutral, or sterically stabilized neutral liposomes were not taken up by these cells.

The aim of our study was to evaluate in vitro cytotoxicity, in vivo biodistribution, pharmacokinetics and efficacy of doxorubicin delivered in cationic liposomes.

2. Materials and methods

2.1. Materials

Egg phosphatidylcholine (EPC), cholesterol (CHOL), and methoxy-polyethylene glycol (M.W. 2000) distearoyl phosphatidylethanolamine (mPEG-DSPE) were purchased from Avanti Polar Lipids, Inc. (Alabaster, AL, USA). 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl tetrazolium bromide (MTT), doxorubicin (DOX), and dimethyl dioctadecyl ammonium bromide (DDAB) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). All other chemicals were analytical or HPLC grade.

2.2. Cell culture

KB, a human oral carcinoma cell line, later identified as a contaminant of HeLa cell line, and L1210, a murine lymphocytic leukemia cell line, were cultured in RPMI 1640 media (Life Technologies Inc., Bethesda, MD, USA), supplemented with 50 μg/mL penicillin, 50 μg/mL streptomycin and 10% fetal bovine serum (FBS) and maintained in a humidified atmosphere containing 5% CO_2 at 37 °C.

2.3. Liposome preparation

Liposomes were prepared by polycarbonate membrane extrusion and then loaded with DOX by pH-gradient driven remote loading (Haran et al., 1993; Pan et al., 2002). Lipid compositions of cationic liposomes and control neutral liposomes were EPC/CHOL/DDAB at molar ratios of 40:40:20 and EPC/CHOL at molar ratio of 60:40, respectively. The composition of PEGylated liposomes was EPC/CHOL/mPEG-DSPE at molar ratios of 55:40:5. Lipid ingredients were dissolved in chloroform ($CHCl_3$) and dried by rotary evaporation and then under vacuum in a round-bottom flask. The lipid film was hydrated with 250 mM ammonium sulfate ($(NH_4)_2SO_4$). The lipid suspension was then extruded five times each through 0.2 and 0.1 μm pore size polycarbonate membranes on a nitrogen-driven Lipex lipid extruder from Northern Lipids Inc. Diafiltration was then used to replace $(NH_4)_2SO_4$ outside the liposomes with phosphate buffered saline (PBS, pH 7.4). DOX solution was mixed with empty liposomes at a drug-to-lipid weight ratio of 1:20 and the mixture was incubated for 30 min at 40 °C with occasional mixing. The liposomes were then purified by size exclusion chromatography on a Sepharose CL-4B column to remove free DOX. Liposome size distribution was determined by dynamic light scattering on a NICOMP Submicron Particle Sizer Model 370 (NICOMP, Santa Barbara, CA, USA). After disruption of the liposomes with ethanol, the amount of entrapped DOX was determined by absorption at 480 nm on a Shimadzu UV–vis spectrophotometer.

2.4. Uptake of cationic DOX liposomes by KB cells

Uptake of cationic liposomes by KB cells was evaluated by fluorescence microscopy and by fluorometry. KB cells were incubated for 2 h at 37 °C with cationic liposomal DOX. The cells were then washed three times with cold PBS and visualized and photographed on a Nikon Eclipse 800 fluorescence microscope. The same experiments were repeated with neutral non-PEGylated and PEGylated liposomes.

The fluorescence of DOX was used to determine the amount of DOX cellular uptake. Cells were incubated with various formulations of DOX for 2 h at 37 °C. Then, the cells were washed with cold PBS and DOX was extracted with ethanol and 20% sodium dodecyl sulfate (SDS). DOX concentration was then measured based on intensity of DOX fluorescence at excitation 480 nm and emission 550 nm on a Perkin-Elmer fluorimeter (LS50B).

2.5. Cytotoxicity analysis

Cytotoxicity of various formulations of DOX was determined by a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolum bromide (MTT) assay, as described previously (Lee and Low, 1995). Briefly, KB and L1210 cells were transferred to 96-well tissue culture plates at 5000 cells per well 24 h prior to drug addition. The culture medium was replaced with medium containing serial dilutions of various drug formulations, including cationic liposomal DOX (cationic L-DOX), neutral liposomal DOX (L-DOX), PEGylated (long circulating) liposomal DOX (PEG-L-DOX), free DOX, and empty cationic liposomes. Following 2 h incubation at 37 °C, the cells were washed twice with PBS and cultured in fresh medium for 72 h, when untreated control wells reached >90% confluence. Then, 20 μL MTT stock solution (5 mg/mL) was added to each well and the plates were incubated for 4 h at 37 °C. Medium was then removed and DMSO was added to dissolve the blue formazan crystals converted from MTT. Cell viability was assessed by absorbance at 570 nm measured on a Biorad microplate reader (Model 450).

2.6. Animals and tumor models

Female 18–22 g DBA/2 mice (Charles River Lab, Wilmington, MA, USA) were injected subcutaneously (s.c.) at the flank with 1×10^7 cells in a volume of 50 μL. Tumors were allowed to grow to an estimated size (based on two-dimensional caliper measurements as described below) of 200–400 mm^3 before initiation of biodistribution studies.

2.7. Pharmacokinetic and biodistribution studies

Mice (in groups of five) received intravenous injections of DOX (5 mg/kg body weight) in various formulations via tail

vein. Blood samples were collected in heparin-treated tubes at various time points (0.083, 0.25, 0.5, 1, 2, 4, 8, 16, and 24 h). Plasma was isolated by centrifugation (10 min at 1500 × *g*) and stored at −20 °C. Tissues specimens were collected, washed, weighed and homogenized in PBS (pH 7.4).

To determine DOX content, 0.2 mL plasma and 0.4 mL tissue homogenate were diluted to 0.5 mL and then 50 μL 20% SDS was added. These samples were vortexed for 30 s after addition of 2.0 mL $CHCl_3$/isopropyl alcohol (1:1, v/v). The resulting samples were frozen overnight, then thawed and centrifuged for 10 min at 1500 × *g*. The organic phase was removed and the amount of associated DOX in fluorescence equivalents was measured by a Perkin-Elmer spectrofluorimeter (SL50B) (excitation/emission at 485:550 nm) (Parr et al., 1997). Doxorubicin extraction efficiency for the assay was determined to be 92.1% for plasma samples, and 88.6% and 86.4% for tumor and lung samples, respectively. Pharmacokinetic parameters were determined using WinNonlin software, including area under the curve (AUC), mean residence time (MRT), total body clearance (CL), volume of distribution (V_d) and plasma half-lives for the distribution ($t_{1/2\alpha}$) and elimination phase ($t_{1/2\beta}$).

2.8. Evaluation of therapeutic efficacy of cationic L-DOX in a murine leukemia model

Mice were inoculated with L1210 cells as described above. On days 5, 9, 13, 17, and 21 following tumor cell implantation, six groups of six animals each received intravenous injections of DOX in various formulations at 5 mg/kg body weight via tail vein, including saline control, free DOX, cationic L-DOX, neutral L-DOX, PEG-L-DOX and empty cationic liposomes. The mice were monitored daily for tumor size and for survival.

2.9. Measurement of tumor size

Tumor dimensions were determined at various time points using a caliper. Tumor volume (mm^3) was calculated using the following formula:

$$\frac{a \times b^2}{2}$$

where *a* is the length and *b* is the width in millimeters. Mice were terminated when tumor volume reached 1500 mm^3 or when the tumor became ulcerated.

2.10. Statistical analysis

Statistical significance on differences between treatment groups were measured by a Student's *t*-test and statistical significance was defined by $p < 0.05$. Data are reported as the mean ± standard deviation (S.D.).

Statistical significance on survival time differences between the treatment groups were measured by a log–rank test and statistical significance was defined by $p < 0.05$.

3. Results

3.1. Properties of cationic L-DOX

Cationic liposomes containing 20% DDAB and control liposomes were prepared by repeated extrusion through polycarbonate membranes with a pore size of 100 nm and DOX were incorporated by remote loading. Properties of the liposomes are summarized in Table 1, including particle size, DOX loading efficiency and zeta potential.

We found that increasing the mol% of cationic lipid resulted in increased accumulation in the lung and reduced plasma half-life of the liposomal formulation. Percentage (20%) of DDAB (cationic lipid) used in this study was selected based on the formulation giving the best overall tumor uptake (tumor level 0.28 μg/g) at 24 h post-injection, since both 10% (tumor level 0.13 μg/g) and 30% (tumor level 0.15 μg/g) DDAB containing liposomes showed reduced tumor uptake.

3.2. Cellular uptake and cytotoxicity of cationic L-DOX

Uptake of cationic L-DOX by KB cells was analyzed by fluorescence microscopy and by fluorometry. As shown in Figs. 1 and 2, cationic liposomes showed much greater cellular uptake compared to control neutral non-PEGylated liposomes and PEGylated liposomes. The results suggest that these liposomes have efficient electrostatic interaction with KB cells.

Cytotoxicity of cationic and control neutral DOX liposomes, PEG-L-DOX, free DOX and empty cationic liposomes was determined in KB and L1210 cells using an MTT assay. The results are summarized in Table 2. The IC_{50} of cationic L-DOX was 7.7 and 6.8 times lower than neutral L-DOX, and 10.8 and 9.1 times lower than PEGylated L-DOX in the two cell lines, respectively. Empty cationic liposomes at the lipid concentration corresponding to that of DOX-containing liposomes at IC_{50} showed no significant cytotoxicity.

3.3. Pharmacokinetic and biodistribution properties

Plasma clearance kinetics of cationic L-DOX was compared to that of free DOX, neutral and PEGylated L-DOX in mice. As shown in Table 3 and Fig. 3, V_d of cationic L-DOX was 15 and 4.6 times greater than that of PEGylated and neutral L-

Table 1
Physical chemical properties of various DOX liposomal formulations ($n = 3$)

DOX formulations	Size (nm)	Loading efficiency (%)	Zeta potential (mV)
PEG-L-DOX	139.7 ± 19.6	96.1 ± 3.77	−24.66 ± 2.69
Cationic L-DOX	109.2 ± 15.7	93.4 ± 3.93	58.62 ± 4.33
Neutral L-DOX	161.2 ± 21.3	94.8 ± 3.01	−4.06 ± 1.54

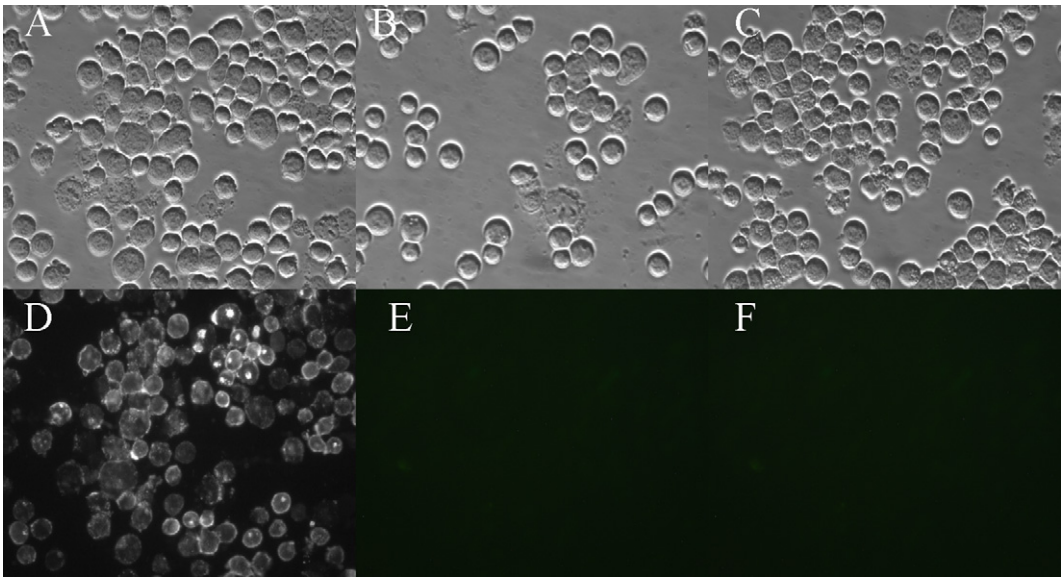

Fig. 1. Uptake of cationic L-DOX, neutral L-DOX and PEGylated L-DOX by KB cells. KB cells were treated with cationic L-DOX, neutral L-DOX, or PEG-L-DOX and were photographed in both fluorescence (dark fields) and phase-contrast (bright fields) modes on a Nikon fluorescence microscope, as described in Section 2. (A) and (D) cells treated with cationic L-DOX; (B) and (E) cells treated with neutral L-DOX; (C) and (F) cells treated with PEG-L-DOX. (A)–(C) are micrographs taken in phase-contrast mode and (D)–(F) are the same fields viewed in fluorescence mode.

Table 2
Cytotoxicity of various DOX formulations to KB and L1210 cells

DOX formulations	IC_{50} (μM)	
	KB	L1210
Cationic L-DOX	1.52 ± 0.30	2.08 ± 0.19
Neutral L-DOX	11.6 ± 1.6	14.0 ± 1.7
PEG-L-DOX	16.4 ± 2.0	19.0 ± 1.9
Free DOX	0.245 ± 0.032	0.109 ± 0.024

Cells were incubated with free DOX, empty cationic liposomes or various formulations of DOX for 2 h at 37 °C. MTT assays were carried out, as described in Section 2. Data are mean ± S.D. ($n = 3$).

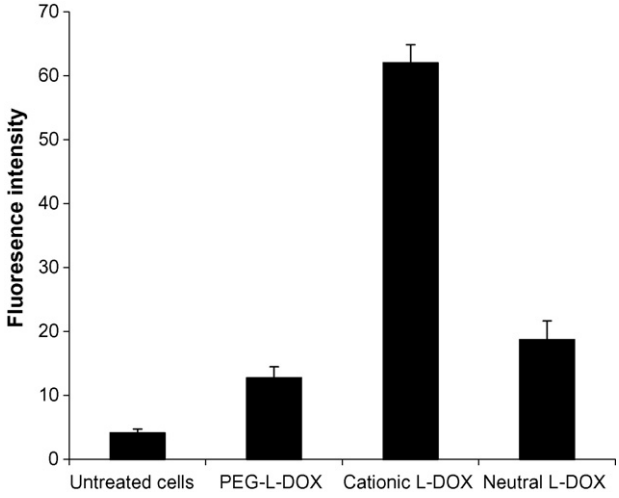

Fig. 2. Uptake of cationic L-DOX by KB cells ($n = 3$). KB cells were treated with various liposomal formulations, as described for Fig. 1. DOX content in cell lysate was measured based on fluorescence at 550 nm with excitation wavelength of 480 nm, as described in Section 2. Data are shown as mean ± S.D.

DOX, respectively. Meanwhile, AUC value of cationic L-DOX in plasma was 96 and 19 times lower than those of the two non-cationic liposomal formulations, respectively. The longer half-lives of neutral and PEGylated liposomal formulations were likely the results of reduced plasma clearance.

To ascertain whether cationic liposomes exhibited greater propensity to accumulate in tumors and lungs, DOX concentrations in tissues of mice receiving various formulations were determined. Fig. 4 and Table 4 indicated that accumulation of cationic L-DOX in lungs was at the highest at 24 h compared to

Table 3
Pharmacokinetic parameters of DOX formulations in mice following i.v. bolus administration

	PEG-L-DOX	Cationic L-DOX	Neutral L-DOX	Free DOX
AUC (0–24 h) (mg mL^{-1} h)	1.16 ± 0.059	0.012 ± 0.002	0.23 ± 0.043	0.010 ± 0.008
MRT (h)	14.72 ± 0.87	1.32 ± 0.34	8.12 ± 1.69	5.40 ± 0.76
$t_{1/2\alpha}$ (h)	0.099 ± 0.021	0.18 ± 0.050	0.21 ± 0.021	0.084 ± 0.006
$t_{1/2\beta}$ (h)	10.2 ± 0.6	1.50 ± 0.41	6.54 ± 1.02	4.20 ± 0.56
CL (mL h^{-1})	0.086 ± 0.004	8.88 ± 1.27	0.44 ± 0.084	10.71 ± 0.96
V_d (mL)	1.27 ± 0.13	19.7 ± 3.1	4.32 ± 1.16	65.4 ± 6.3

The values are shown as mean ± S.D. ($n = 5$).

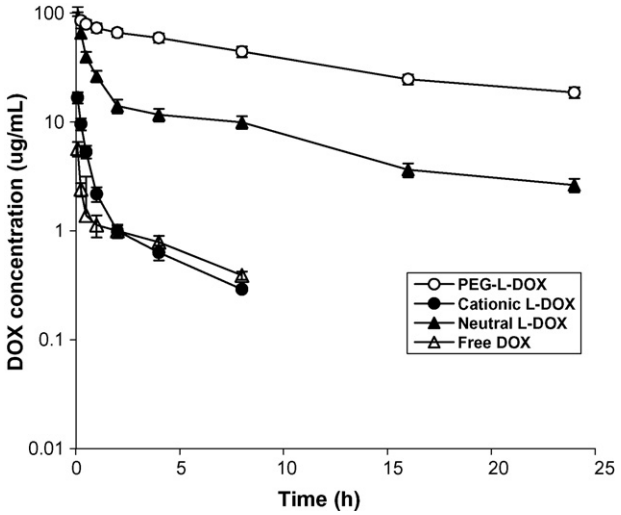

Fig. 3. Plasma concentration vs. time curves for various DOX formulations. Formulations were administered via i.v. tail-vein injection into DBA/2 mice at a dose of 5 mg/kg DOX. Data are shown as mean ± S.D. ($n = 5$).

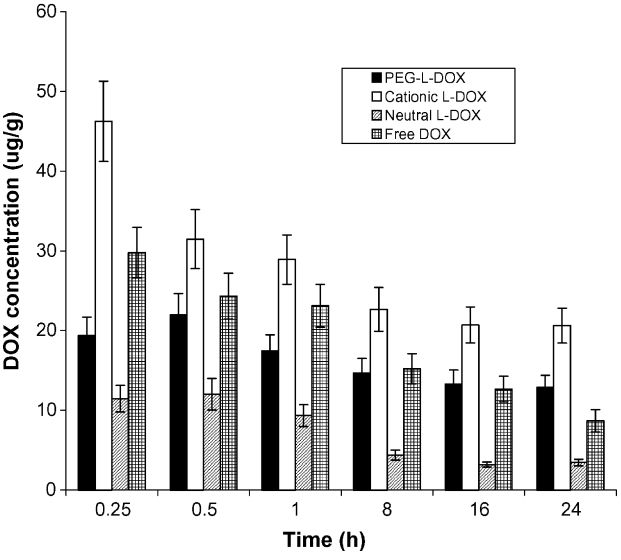

Fig. 4. Time-dependent changes in DOX concentration in lung following injection of DOX liposomes. Mice in groups of five received i.v. injections of 5 mg/kg of DOX. Tumor DOX levels were determined by its fluorescence, as described in Section 2. Data are shown as mean ± S.D.

Table 4
Accumulation of DOX various formulations in tumor and lung at 24 h

	AUC (mg mL^{-1} h)	
	Tumor	Lung
PEG-L-DOX	0.30 ± 0.056**	0.33 ± 0.051
Cationic L-DOX	0.27 ± 0.035*	0.59 ± 0.101*
Neutral L-DOX	0.16 ± 0.021	0.11 ± 0.016
Free DOX	0.14 ± 0.027	0.35 ± 0.067

Mice in groups of five received i.v. injections of 5 mg/kg of DOX in various formulations. DOX levels were determined, as described in Section 2. Data are shown as mean ± S.D.

* Vs. various DOX formulations, $p < 0.05$. Data are shown as mean ± S.D.

** Vs. cationic L-DOX, $p > 0.05$.

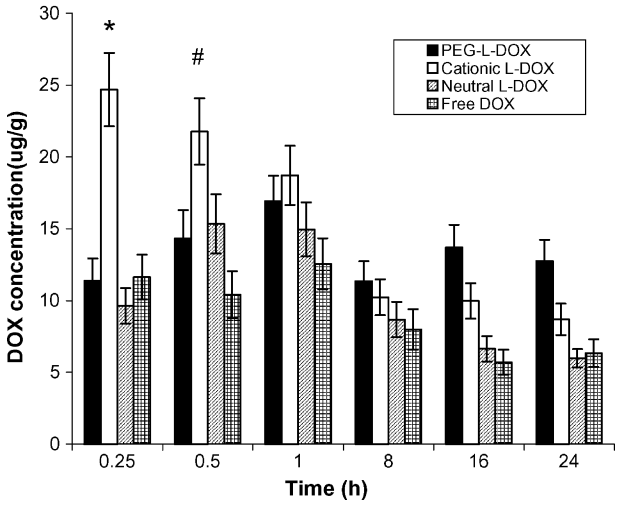

Fig. 5. Time-dependent changes in DOX concentration in tumor following injection of various liposomal formulations. Mice in groups of five received i.v. injections of 5 mg/kg of DOX. Tumor DOX levels were determined by fluorometry, as described in Section 2. Data are shown as mean ± S.D. $^{*}p < 0.01$, $^{\#}p < 0.05$: vs. various DOX formulations.

various formulations ($p < 0.05$). Fig. 5 and Table 4 indicated that accumulation of cationic L-DOX in tumors was higher than that of free DOX and neutral L-DOX at 24 h ($p < 0.05$). However, the AUC value of PEG-L-DOX in tumors was similar to that of cationic L-DOX at 24 h ($p > 0.05$).

The therapeutic efficacy of cationic L-DOX was evaluated in a murine leukemia model. Mice were treated in groups of six each with five i.v. injections of the various formulations, as described in Section 2. Mice survival rates are presented in Fig. 6. Median survival time for the six groups of mice were 77.5 days (cationic L-DOX), 67.5 days (PEG-L-DOX), 42.5 days (free DOX), 42.5 days (neutral L-DOX), 32.5 days (empty cationic liposomes) and 23 days (PBS control), respectively. Data on animal survival (Fig. 6) indicated that cationic L-DOX

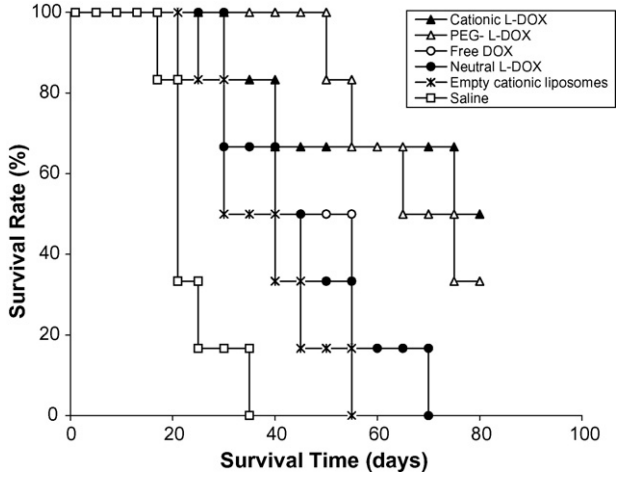

Fig. 6. Effect of cationic L-DOX treatment on the survival of DBA/2 mice carrying L1210 tumor. DBA/2 mice inoculated subcutaneously with L1210 cells were treated with cationic L-DOX, PEG-L-DOX, free DOX, neutral L-DOX, empty cationic liposomes and saline control, as described in Section 2. Animal survival was recorded from the day of inoculation.

was significantly more effective than neutral L-DOX and free DOX ($p < 0.01$ by log–rank test). The mice treated with cationic L-DOX and PEGylated L-DOX showed a significantly greater increase-in-lifespan (ILS) than mice treated with other formulations. Furthermore, the survival time of mice receiving cationic L-DOX was greater than that of mice receiving long circulation L-DOX mice ($p < 0.05$ by log–rank test). These results suggest that cationic L-DOX exhibit enhanced therapeutic efficacy over PEGylated liposomes in this model.

4. Discussion

The vascular network is a highly accessible target for tumor therapy. Cationic liposomes have been shown to accumulate selectively in angiogenic tumor endothelial cells. Therefore, they are promising as a drug carrier for delivery to the tumor endothelium.

This study reports the characterization of cationic liposomal formulations of DOX. Enhancement in both cellular uptake and in vitro cytotoxicity was demonstrated. This might be due to the ability of cationic liposomes to interact with cells via electrostatic interaction, to induce internalization by endocytosis, and to facilitate drug release into the cytosol by endosomal escape. These properties of cationic liposomes have been widely known in the context of nucleic acid delivery. Cationic lipids themselves have been shown to exhibit cytotoxicity at elevated concentration. It is, therefore, possible that the observed increase in cytotoxicity by cationic liposomal DOX was partly contributed by effect of combination of cationic lipids and chemotherapy agents.

Pharmacokinetic studies on DOX liposomal formulations indicated that cationic liposomes are rapidly cleared from circulation and distributed into tissues. As shown in Table 3, V_d value of cationic L-DOX was higher, while the AUC value was lower than other liposomal formulations, which meant that cationic L-DOX exhibited accelerated drug distribution in tissues. The cationic charge of the liposomes are likely to increase their interaction with endothelium of a variety of tissues as well as with plasma protein, although there was clearly selectivity in the tissue distribution. In fact, it was shown that cationic L-DOX efficiently accumulated in the lung, which contains the first capillary bed encountered by the liposomes following i.v. administration. This suggested that cationic liposomes have potential utility in lung-targeted therapeutic delivery. On the other hand, the overall tumor uptake was not significantly different between cationic L-DOX and long circulating PEG-L-DOX at 24 h ($p > 0.05$), both of which exhibit higher DOX accumulation in tumors than free DOX and neutral liposomes. Campbell et al. (2002) and Krasnici et al. (2003) reported similar findings that total localization in the tumor was not dependent on cationic liposomal formulation. However, significant differences between cationic L-DOX and other formulations were shown at the 30 min time point ($p < 0.05$). The results suggested that cationic distributed into tumors rapidly. A possible mechanism for this kinetic difference is that cationic liposomes were taken up by tissues due to their charge and resulting electrostatic interactions. In contrast, PEGylated liposomes, which are slightly negatively charged, have reduced clearance rate from the ciruculation and were able to localize in tumors via enhanced permeation and retention (EPR) effect. Compared to electrostatic interactions, EPR effect depends on extravasation, which is a relatively slow process.

Data from in vivo survival analyses using the L1210 murine leukemia model showed a significant therapeutic advantage for cationic L-DOX over free DOX, neutral L-DOX and PEGylated L-DOX. Enhancement of median survival time and survival rates might have resulted from differences in the cytotoxicties of various liposomal formulations. Data shown in Table 2 indicated that IC_{50} value was lowered for the cationic liposomes compared to PEGylated and neutral liposomes. A possible mechanism is that cationic liposomes can enter cells via charge mediated binding, endocytosis, and endosomal drug release. Accelerating intracellular drug release has been shown to be critical for the therapeutic activity of liposomal doxorubicin, as shown in studies on thermosensitive liposomes (Kong et al., 2000). In contrast to thermosensitive liposomes, which required local application of heat from an external source, cationic liposomes are triggered to release their content upon cellular interactions.

Possible mechanisms by which cationic liposomes selectively target activated tumor endothelium include receptor-mediated endocytosis (Thurston et al., 1998) and charge-dependent binding and uptake by a potentially altered glycocalyx on endothelial cells of tumor microvessels (Krasnici et al., 2003). Paclitaxel cationic liposomes have previously been shown preferential targeting of tumor vessels compared to surrounding normal tissue (Strieth et al., 2004; Schmitt-Sody et al., 2003). This study, however, is the first to report greater in vitro cytotoxicity and in vivo efficacy of cationic liposomes encapsulating DOX.

5. Conclusion

A cationic liposomal DOX formulation was synthesized and evaluated. The formulation was efficiently taken up by and showed enhanced cytotoxicity in KB and L1210 cells. Pharmacokinetic and biodistribution studies indicated that cationic L-DOX showed rapid clearance from the blood, rapid accumulation in the tumor, and increased therapeutic efficacy. Further studies will focus on understanding of mechanisms of the observed antitumor activity by drugs delivered via cationic liposomes.

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