ELSEVIER

International Journal of Pharmaceutics 146 (1997) 31–39

international journal of pharmaceutics

Comparative pharmacokinetics and antitumor efficacy of doxorubicin encapsulated in soybean-derived sterols and poly(ethylene glycol) liposomes in mice

Xian-Rong Qi[a], Yoshie Maitani[b,*], Tsuneji Nagai[b], Shu-Li Wei[a]

[a] *School of Pharmaceutical Sciences, Beijing Medical University, Beijing, 100083, China*
[b] *Department of Pharmaceutics, Hoshi University, Ebara 2-4-41, Shinagawa-ku, Tokyo 142, Japan*

Received 24 June 1996; revised 29 August 1996; accepted 24 September 1996

Abstract

The blood clearance, tissue uptake and antitumor efficacy of doxorubicin (DOX) encapsulated in two different types of liposomes against hepatoma 22 (H22) and sarcoma 180 (S180) tumors were examined in mice. Liposomes were composed of dipalmitoylphosphatidylcholine (DPPC) and soybean-derived sterol mixture (SS) at a 7:4 molar ratio (DPPC/SS-liposomes), a new stabilizer like cholesterol, and 6 mol% distearoylphosphatidylethanolamine (DSPE) derivatized with poly(ethylene glycol) (PEG) (DPPC/SS/PEG-liposomes). Pharmacokinetic analysis of drug disposition was based on the areas under the curve (AUC) for liposome-encapsulated DOX uptake per gram tissue up to 24 h following i.v. injection. The highest tissue AUC values with both liposome types were obtained in spleen and liver. The serum AUC value of DPPC/SS/PEG-liposomes was 1.3 times higher than that of DPPC/SS-liposomes ($P < 0.05$). These findings indicate that the encapsulation of DOX in either DPPC/SS- or DPPC/SS/PEG-liposomes markedly prolonged the blood circulation time. The antitumor efficacy of DOX encapsulated in liposomes was compared with that of the free drug at two doses, 5 and 10 mg/kg. The antitumor efficacy of DOX encapsulated in DPPC/SS- and DPPC/SS/PEG-liposomes was different between the H22 and S180 tumor. DPPC/SS-liposomes were significantly more active against the H22 tumor than the free drug and the DPPC/SS/PEG-liposomes markedly more active (ILS: 32.1 and 97.7%, respectively, $P < 0.001$), reflecting long circulation. The antitumor efficacy of the DPPC/SS/PEG-liposomes against S180 tumor-bearing mice was significantly high but that of DPPC/SS-liposomes was not, in comparison with free DOX.

Keywords: Liposomes; Doxorubicin; Poly(ethylene glycol); Soybean-derived sterols; Hepatoma 22; Sarcoma 180

* Corresponding author. Tel.: +81 3 54984635; fax: +81 3 54985783.

0378-5173/97/$17.00
PII S0378-5173(96)04753-9

1. Introduction

Doxorubicin (DOX) is a potent antineoplastic agent active against a wide range of human neoplasms. However, administration of this drug is associated with severe acute toxicities (including myelosuppression and gastrointestinal toxicity) as well as a cumulative dose-limiting cardiotoxicity. Many reports in various animal models consistently indicate the usefulness of liposome encapsulation to ameliorate both the acute and chronic toxic side effects of DOX (Olson et al., 1982; Mayer et al., 1989).

The use of liposomes as drug delivery vehicles requires extended blood residence time to allow optimal delivery to target tissue. The tumor targeting can be improved by using liposomes consisting of the ganglioside GM1 (Unezaki et al., 1993) or amphipathic poly(ethylene glycol) (PEG) (Huang et al., 1992; Maruyama et al., 1992) to avoid uptake by the reticuloendothelial system (RES).

Also, the stabilization effect of component on the liposome is important for a long circulation time in blood. Small unilamellar liposomes composed of cholesterol (Ch) and a solid-phase (at 37°C) phospholipid have been shown to be cleared slowly from the circulation, compared to liposomes composed of fluid phospholipids (Allen et al., 1989; Hwang et al., 1980). Animal cells mainly contain Ch and plants contain sterols. Sterols have been reported to show a function similar to that of Ch in membranes (Demel and De Kruijff, 1976). Soybean-derived sterols (SS) are the residue after the soybean oil has been extracted. The SS used in this study was a mixture of β-sitosterol (49.9%), campesterol (29.1%), stigmasterol (13.8%), and brassicasterol (7.2%). We have reported that SS stabilized the dipalmitoylphosphatidylcholine (DPPC) liposomes of reverse-phase evaporation vesicle (REV), that the multilamellar vesicle (MLV) encapsulated calcein in vivo and vitro more effectively compared to Ch (Qi et al., 1995a,b; Muramatsu et al., 1994), and that the stabilizing effect of SS on DPPC-liposomes was the greatest at a molar ratio of DPPC and SS of 7:4 (Qi et al., 1995a). The SS in DPPC/SS-liposomes may stabilize the liposomes by making the DPPC bilayer film rigid above its gel-liquid crystalline phase transition temperature of liposomes, reducing the film interaction between each liposome and suppressing the causes of flocculation. The rigidity of liposomes containing SS might inhibit opsonization; therefore, the liposomes would tend to remain in the blood circulation.

Herein, we examined the effects of DOX encapsulated in two types of DPPC-liposomes containing SS in the same molar ratio (7:4), but only one containing PEG, on blood clearance, tissue uptake and antitumor efficacy in mice.

2. Materials and methods

2.1. Materials

DPPC and PEG- distearoylphosphatidylethanolamine (PEG-DSPE) with PEG at an average molecular weight of 2000, were purchased from NOF (Tokyo, Japan). DOX was purchased from Sigma (St. Louis, MO). SS was provided by Ryukakusan (Tokyo, Japan). All other chemicals used were of reagent grade.

2.2. Preparation of liposomes

Liposomes were prepared from DPPC, SS (DPPC/SS-liposomes, 7:4, molar ratio) and 6 mol% PEG-DSPE (DPPC/SS/PEG-liposomes, 7:4:6%, molar ratio) by the REV method (Szoka and Papahadjopoulos, 1978). Briefly, the lipid mixture was dissolved in chloroform/isopropyl ether (1:1, v/v) and 300 mM citric acid (pH 4.0) was added. The mixture was sonicated with a bath-type sonicator to give a w/o-type emulsion. Then the organic solvents in the emulsion were evaporated to form a REV suspension. Liposomes were successively extruded through a polycarbonate membrane (Nuclepore, USA) with a pore size of 200 nm at about 60°C to make small liposomes. The size distribution of liposomes estimated was quiet homogeneous and the mean diameter was about 134 nm measured by a Nicomp 370 submicron particle analyzer (Pacific Scientific, CA).

2.3. DOX encapsulation

DOX was encapsulated in DPPC/SS- and DPPC/SS/PEG-liposomes by the pH gradient method (Mayer et al., 1990). Briefly, the pH of the liposomes suspension (initially pH 4.0) was raised to pH 7.8 with 1 N NaOH solution. The liposome suspension was subsequently heated to 60°C for 5 min. The liposomes were then mixed with a preheated (60°C) solution of DOX in Hepes buffer (pH 7.8). This mixture was incubated with periodic mixing for 15 min at 60°C. The resulting preparation was finally passed through a Sephadex G-50 column with sodium citrate buffer (pH 7.8) in all fractions.

2.4. Animals and tumor models

Male ddY mice each weighing about 20–30 g (6 weeks old) were purchased from Saitama Experimental Animal Supply (Saitama, Japan). Male Kun-Ming mice each weighing about 16–24 g (6 weeks old) were purchased from the animal center of Beijing Medical University (Beijing, China).

Murine carcinoma of Hepatoma 22 (H22) cells and murine Sarcoma 180 (S180) cells were maintained by weekly transplantation of the tumor cells into the peritoneal cavity and the right axillary subcutaneous tissue of male Kun-Ming mice, respectively. To obtain the suspensions of tumor cells for transplantation, the ascites fluid containing H22 cells was diluted to about 10^8 cells/ml with sterile saline, and 0.2 ml of the diluted suspension was injected intraperitoneally per mouse. S180 cells were prepared by the addition of 3 ml of sterile saline per g of tumor solid and subsequently homogenized and suspended, and 0.2 ml of the tumor cells suspension was injected subcutaneously per mouse.

2.5. Tissue distribution studies

Liposomal DOX was injected via a tail vein at a dose of 5 mg DOX/kg to groups of three or four male ddY mice (20–30 g, body weight). At indicated times after injection the mice were killed by cervical dislocation, blood was collected by heart puncture, and the liver, heart, lungs, kidneys, and spleen were immediately removed. Tissues were lightly blotted to remove any excess blood and weighed. These tissues, as well as 0.2 ml serum were homogenized and extracted with chloroform/methanol (4:1, v/v), the extracts were then subjected to high performance liquid chromatography (HPLC) assay (Shinozawa et al., 1980).

The HPLC system consisted of a shimadzu LC-5A high-pressure pump, a shimadzu RF-535 fluorescence detector (EX, 470 nm; EM, 585 nm) and a YMC-Pack C_{18} column. The mobile phase was methanol/water/acetic acid (50:45:5, v/v/v) and a flow rate of 1.0 ml/min was used. Measurements were made using the ratio of the peak area to that of an internal standard (daunomycin).

2.6. Evaluation of antitumor activity

Mice, in groups of ten, were implanted intraperitoneally 4×10^7 H22 viable tumor cells suspended in 0.2 ml sterile saline. Treatment was initiated 1 day following implantation of tumor cells and was given as a single i.v. dose via the tail vein. The animals were treated with free or liposomal DOX at 10 mg/kg. The control group was treated with sterile saline. Mice were weighed on the day before tumor implantation, and weights were recorded twice weekly. Survival time was recorded in days following tumor implantation. Antitumor activity was evaluated by comparing the mean survival time of the treated animals (T) with that of the control animals (C), i.e. by calculation of the increase in life span (ILS), $(T/C\text{-}1) \times 100$ (%) (Huang et al., 1992).

Concerning S180, the tumor cell suspension was implanted subcutaneously of 1×10^7 viable tumor cells suspended in 0.2 ml sterile saline. Treatment was initiated 1 or 7 days following implantation of S180 tumor cells and was given at 5 and 10 mg/kg, respectively, as a single i.v. dose via the tail vein. The control group was treated with sterile saline. Antitumor activity was evaluated in terms of the tumor growth rate. The tumor size was measured with slide calipers and the mice were weighed twice weekly after implantation. Each size was calculated by approximation of the tumor solid to an ellipsoid, i.e. using the equation, $s = k \times a \times b \times c$, where a, b and c are length,

Table 1
Tissue AUC values after i.v. injection of DOX encapsulated in DPPC/SS- and DPPC/SS/PEG-liposomes in mice at a dose of 5 mg/kg

Formulation of liposomes	Tissue AUC[a] (h μg/g)						
	Serum[b]	Heart	Liver	Spleen	Kidney	Lung	Tumor
DOX[c]	1.0	63.2	168.8	178.1	146.1	106.4	18.4
DPPC/SS	459.7*	27.4	297.6	184.9**	30.7	11.4	—
DPPC/SS/PEG	614.5*	20.4	287.2	115.8**	31.0	11.6	—

[a] AUC values were calculations for 0–24 h ($n = 3$–4).
[b] Serum AUC is given as h μg/ml ($n = 3$–4).
[c] Data from Unezaki et al., 1995.
* $P < 0.05$, ** $P < 0.01$.

width and height (cm), respectively, s is size (cm^3) and k a constant. Finally, the mice were killed at 14 days following tumor implantation, and their tumor solids were weighed. The growth-inhibitory effects (GIE) of the liposomal DOX against S180 cells were evaluated by comparing the mean tumor weight (g) of the treated animals (T) with that of the control animals (C), $(1 - T/C) \times 100$ (%).

3. Results

3.1. Tissue distribution of DPPC/SS- and DPPC/SS/PEG-liposomes

Fig. 1 shows DPPC/SS- and DPPC/SS/PEG-liposomes remaining in serum and uptake by heart, liver, spleen, kidneys, and lungs. Table 1 shows the calculated pharmacokinetic parameters. The amount of DPPC/SS/PEG-liposomes remaining in serum 24 h after injection was almost four times higher than that of DPPC/SS-liposomes (Fig. 1A). The serum AUC of DPPC/SS/PEG-liposomes was 1.3 times higher than the serum AUC of DPPC/SS-liposomes ($P < 0.05$, Table 1). The uptake in liver, which is one of the tissues rich in cells of the RES, was higher for DPPC/SS-liposomes, compared to DPPC/SS/PEG-liposomes at the initial stage after injection, and was similar after 2 h of i.v. injection (Fig. 1B). The calculated AUC of DPPC/SS-liposomes was slightly greater than that of DPPC/SS/PEG-liposomes in the liver (Table 1). The uptake in spleen, which is another tissue rich in cells of the RES, was higher for DPPC/SS-liposomes, compared to DPPC/SS/PEG-liposomes (Fig. 1C). The calculated AUC of DPPC/SS-liposomes was 1.6-fold greater compared to the AUC of DPPC/SS/PEG-liposomes in spleen ($P < 0.01$, Table 1).

The liposome uptake by other normal tissues (heart, kidneys and lungs) was much lower than the uptake by RES-rich tissues for both DPPC/SS- and DPPC/SS/PEG-liposomes (Fig. 1D, E and F) and the AUC values were similar (Table 1).

3.2. Antitumor activity

Table 2 shows the antitumor efficacy of liposomal DOX as measured in the murine H22 and S180 tumor models. The liposomal forms displayed stronger antitumor activity than the free drug, i.e. the ILS values of the liposomal forms were significantly greater. The ILS value was significantly greater for DPPC/SS/PEG-liposomes (97.7%) than the DPPC/SS-liposomes (32.1%) ($P < 0.001$). This indicated that the DPPC/SS/PEG-liposomes are more effective therapeutically than the DPPC/SS-liposomes. This is illustrated by representative survival curves shown in Fig. 2.

Fig. 3A shows the tumor growth rate in terms of mean tumor size (cm^3) for injection on day 1 following tumor implant at 5 mg/kg, Fig. 3B for injection on day 1 following tumor implant at 10 mg/kg, and Fig. 3C for injection on day 7 following tumor implant at 5 mg/kg. These findings

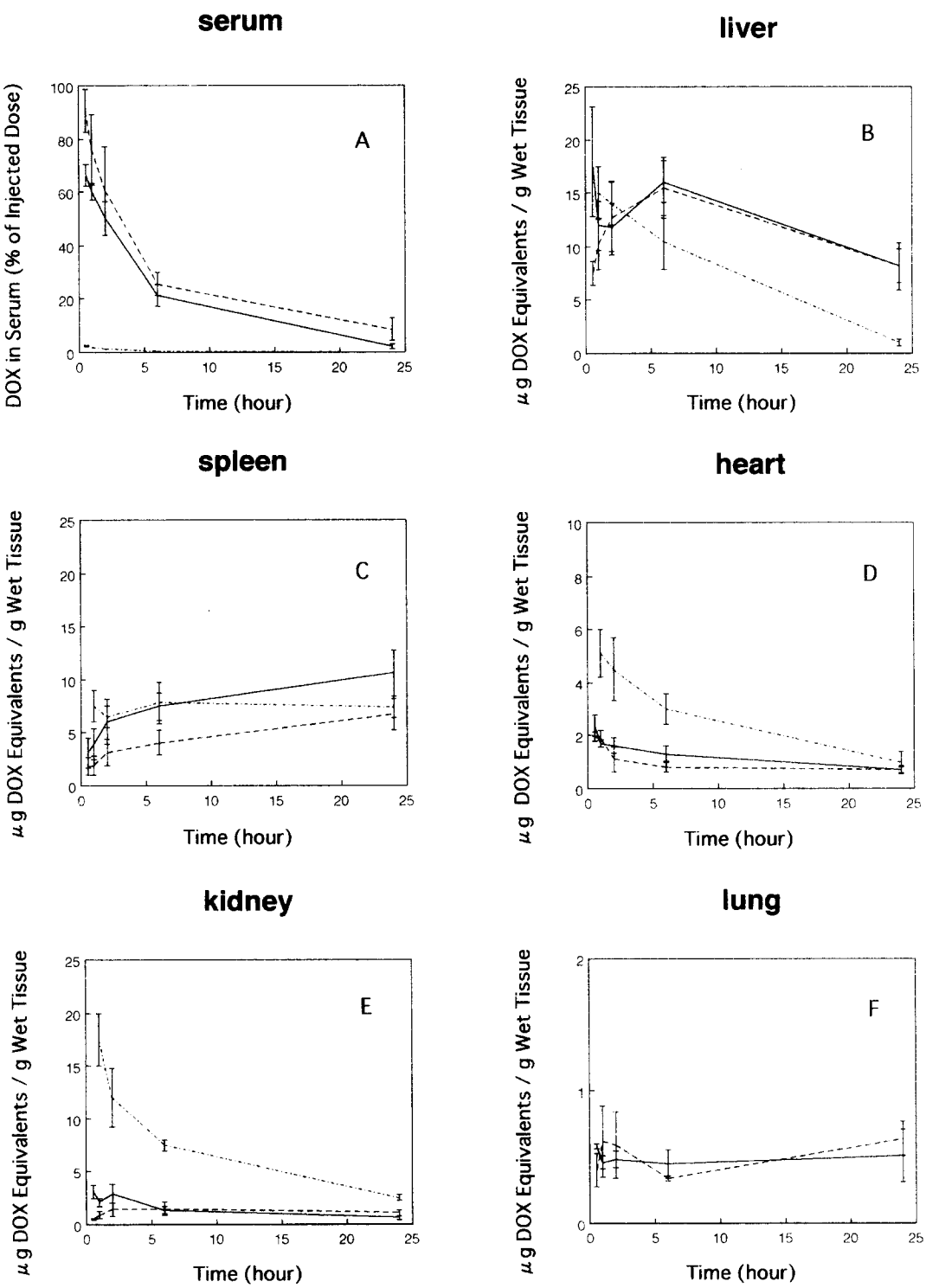

Fig. 1. Drug levels in various tissues after i.v. injection of DOX encapsulated in DPPC/SS-liposomes (——), DOX encapsulated in DPPC/SS/PEG-liposomes (- - -) and free DOX (– - –) in mice at a dose of 5 mg/kg. Results are given as means ± S.D. ($n = 3$–4). The values of free DOX were obtained from Unezaki et al. (1995).

Table 2
Comparative efficacy of DOX encapsulated in liposomes against H 22 tumor and S 180 tumor in mice

Treat material	Cancer model	DOX dose (mg/kg)	Injected day	No. of mice/ group	Survival days (mean ± S.D.)	Tumor weight (g) (mean ± S.D.)	ILS[a] (%)	GIE[b] (%)
Saline	H22	—	1	10	13.1 ± 2.2	—	—	—
Free DOX	H22	10	1	10	14.5 ± 3.1	—	10.7	—
DPPC/SS	H22	10	1	10	17.3 ± 2.6	—	32.1	—
DPPC/SS/PEG	H22	10	1	10	25.9 ± 4.9	—	97.7	—
Saline	S180	—	1	6	—	1.57 ± 0.76	—	—
Free DOX	S180	5	1	6	—	1.53 ± 0.50	—	2.5
Free DOX	S180	10	1	6	—	1.34 ± 0.81	—	14.6
Free DOX	S180	5	7	8	—	1.37 ± 0.97	—	12.7
DPPC/SS	S180	5	1	6	—	2.11 ± .0.49	—	−34.4
DPPC/SS	S180	10	1	6	—	1.48 ± 0.70.	—	5.7
DPPC/SS	S180	5	7	9	—	1.45 ± 1.00	—	7.6
DPPC/SS/PEG	S180	5	1	6	—	0.90 ± 0.56	—	42.7
DPPC/SS/PEG	S180	10	1	6	—	0.88 ± 0.50	—	43.9
DPPC/SS/PEG	S180	5	7	7	—	0.84 ± 0.53	—	46.5

[a] ILS, increase in life span ((T/C-1) × 100 (%)), where T and C represent the mean survival time (days) of the treated and control animals, respectively.
[b] GIE, the growth-inhibitory effects ((1-T/C) × 100 (%)), where T and C represent the mean tumor weight (g) of the treated and control animals, respectively.

showed that the maximal tumor growth inhibition was obtained from DPPC/SS/PEG-liposomes. The DPPC/SS-liposomes did not have a strong tumor growth inhibition effect. The mean GIE values were greater for the DPPC/SS/PEG-liposomes than the free DOX and DOX encapsulated in DPPC/SS-liposomes (Table 2).

4. Discussion

4.1. Tissue distribution

Studies using cancer chemotherapeutic agents such as DOX encapsulated in liposomes have generally shown reduced toxicity and in some cases enhanced therapeutic efficacy compared to administration of free drug in some animal tumor models (Olson et al., 1982; Mayer et al., 1989; Unezaki et al., 1993; Huang et al., 1992). Studies on the tissue distribution of liposome uptake after i.v. administration have indicated that size, membrane rigidity and surface charge of liposomes are among the major factors that determine the clearance of circulating liposomes from the blood.

We have reported that SS stabilized the DPPC-liposomes of REV and the MLV encapsulated calcein in vivo and vitro (Qi et al., 1995a,b; Muramatsu et al., 1994) and the stabilizing effect

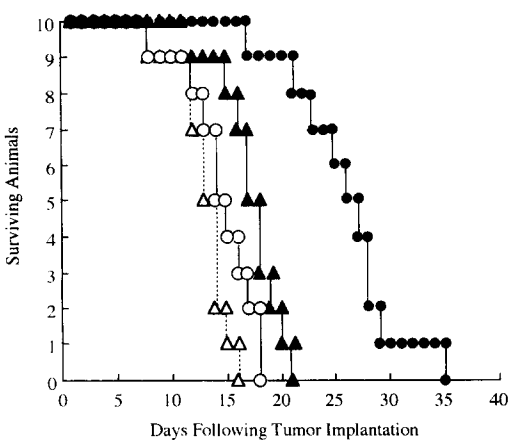

Fig. 2. Effects of free DOX and liposomal DOX on survival of mice implanted with H22 cells at a dose of 10 mg/kg ($n = 10$). - - △ - -, saline control; – ▲ –, DPPC/SS-liposomes; – ○ –, free DOX; – ● –, DPPC/SS/PEG-liposomes.

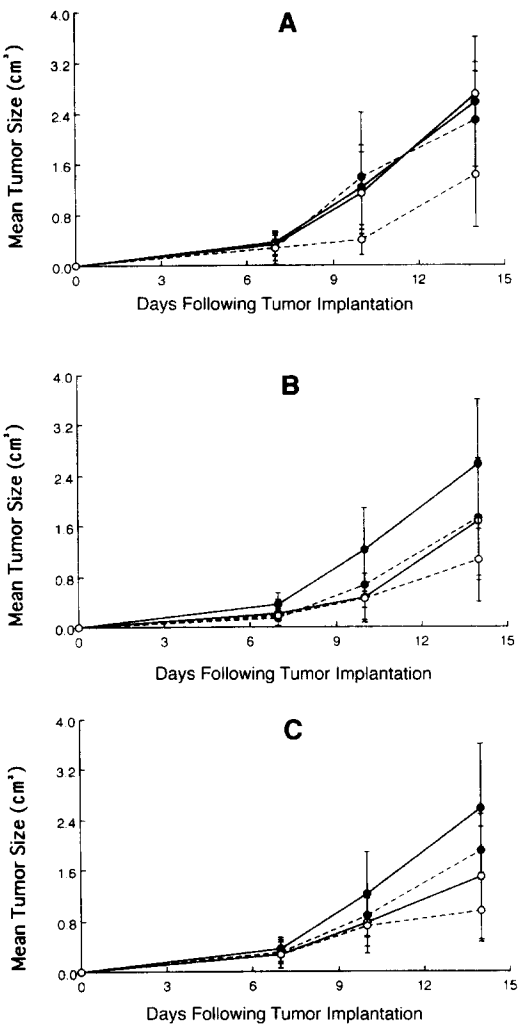

Fig. 3. Effects of free DOX and liposomal DOX on growth of S180 tumor. (A) Injection on day 1 following tumor implantation at 5 mg/kg. (B) Injection on day 1 following tumor implantation at 10 mg/kg. (C) Injection on day 7 following tumor implantation at 5 mg/kg. – ● –, saline control; - - ● - -, free DOX; – ○ –, DPPC/SS-liposomes; - - ○ - -, DPPC/SS/PEG-liposomes. Results are given as means ± S.D. ($n = 6$–9).

of SS on DPPC-liposomes was the greatest at a molar ratio of DPPC and SS of 7:4 (Qi et al., 1995a), at which the membrane rigidity was the greatest, reflecting long circulation (Qi et al., 1996).

Unezaki et al. reported the AUC value calculated for 0–24 h of free DOX in blood, heart,

liver, spleen, kidneys, lungs after i.v. injection in tumor-bearing mice at a dose of 5 mg/kg (Unezaki et al., 1995). Compared with their study, the DPPC/SS- and DPPC/SS/PEG-liposomes had a higher blood, liver and spleen AUC values and lower heart, lungs and kidneys AUC values than those of free DOX (Table 1). Thus, these findings indicate that the encapsulation of DOX in either DPPC/SS- or DPPC/SS/PEG-liposomes are markedly effective in prolonging blood circulation time and may reduce heart toxicity. The DPPC-liposomes containing SS (7:4) were superior in prolonging the blood circulation time to DPPC/Ch (7:2)-liposomes significantly ($P < 0.05$) (Qi et al., 1995a,b; Muramatsu et al., 1994). The present findings also indicate that liposomes which contain PEG have a markedly longer circulation time than the DPPC/SS-liposomes without PEG.

4.2. Antitumor activity

Antitumor activity studies, with a single injection on day 1, indicated that DPPC/SS- and DPPC/SS/PEG-liposomes encapsulating DOX were effective in inhibiting H22 tumor growth (Table 2). The survival period reflects not only the antitumor activity but also the toxicity of the drug. The 10 mg/kg dose of free DOX resulted in a slight but not significant increase in survival time compared to the controls (10.7%, $P > 0.05$) (Table 2). This is possibly because of non-specific toxicity. These findings are in accordance with earlier observations that DOX liposomes are less toxic than free DOX (Huang et al., 1992; Forssen and Tokes, 1979; Gabizon and Papahadjopoulos, 1988). All the earlier studies on liposome encapsulated DOX have shown that a decrease in toxicity is the basis for the observed increase in the therapeutic index (Huang et al., 1992; Forssen and Tokes, 1979; Gabizon and Papahadjopoulos, 1988).

The therapeutic studies indicated that PEG-containing liposomes encapsulating DOX were effective in inhibiting S180 tumor growth, whereas liposomes lacking PEG were ineffective (Table 2). Comparison of the two liposome formulations, the DPPC/SS/PEG-liposomes were significantly effective on the decrease of the mean tumor weight and the mean tumor size of mice bearing S180 tumor compared to DPPC/SS-liposomes and free DOX, showing that sterically stabilized liposomes were more effective therapeutically. As Table 2 and Fig. 3 show, the mean tumor weight and the mean tumor size in both the groups of free DOX and liposomal DOX injected at day 7 were comparatively smaller than those in the groups injected at day 1 following S180 cells implant at the same dose (5 mg/kg). This is in agreement with the report by Huang et al. (1992) that the maximal antitumor activity against the colon 26 tumor was obtained when there was a delay of 6–9 days between the injection of the tumor cells and liposomes for a single dose. This indicated that the liposome-derived antitumor effect is not due to the systemic release of the drug but is due to its release from the liposomes that localize within the growing tumor mass (Huang et al., 1992). The delay is probably due to the necessary development of new vasculature around the tumor mass, which allows for increased localization of liposomes (Hori et al., 1990).

In many types of tumors, the tumor bed is well vascularized and may be better vascularized than some normal tissue (Jain, 1987), increasing the opportunity for liposome delivery to tumor regions with efficient blood circulation. Furthermore, liposomes may escape to the extravascular space surrounding tumor cells, due to defects in the tumor vasculature and discontinuous or absent basement (Jain, 1987). This may be one of the reasons why the DPPC/SS- and DPPC/SS/PEG-liposomes are more effective than free DOX against tumors, reflecting long circulation (Table 1).

The present findings show that DPPC/SS/PEG-liposomes were more effective in prolonging the circulation time in blood and in increasing survival of tumor-bearing mice and inhibiting the tumor growth than the DPPC/SS-liposomes. This is because PEG can prevent the uptake of the liposomes by spleen. The mechanism by which PEG prevents the uptake of liposomes by spleen may be related to its ability to impart a hydrophilic surface to the liposomes. This bulky, hydrophilic surface may prevent opsonization of the surface of the liposomes with plasma protein

involved in the recognition and uptake of liposomes (Woodel and Lasic, 1992).

5. Conclusions

The present findings indicate that encapsulation of DOX in either DPPC/SS- or DPPC/SS/PEG-liposomes is markedly effective in prolonging the blood circulation time and therefore is more active against the H22 tumor than the free drug; the DPPC/SS/PEG-liposomes are significantly more active against the H22 tumor than the DPPC/SS-liposomes. The antitumor efficacy of DPPC/SS/PEG-liposomes against S180 tumor-bearing mice is significantly higher than that of free DOX, but not that of DPPC/SS-liposomes.

References

Allen, T.M., Hansen, C. and Rutledge, J., Liposomes with prolonged circulation times: factors affecting uptake by reticuloendothelial and other tissues. *Biochim. Biophys. Acta*, 981 (1989) 27–35.

Demel, R.A. and De Kruijff, B., The function of sterol in membranes. *Biochim. Biophys. Acta*, 457 (1976) 109–132.

Forssen, E.A. and Tokes, J.A., In vitro and in vivo studies with adriamycin liposomes. *Biochem. Biophys. Res. Commun.*, 91 (1979) 1295–1301.

Gabizon, A. and Papahadjopoulos, D., Liposome formulation with prolonged circulation time in blood and enhanced uptake by tumors. *Proc. Natl. Acad. Sci. USA*, 85 (1988) 6949–6953.

Hori, K., Suzuki, M., Tanda, S. and Sito, S., In vivo analysis of tumor vascularization in the rat. *Jpn. J. Cancer Res. Clin. Oncol.*, 81 (1990) 279–288.

Huang, S.K., Mayhew, E., Gilani, S., Lasic, D.D. Martin, F.J. and Papahadjopoulos, D., Pharmacokinetics and therapeutics of sterically stabilized lipsomes in mice bearing c-26 colon carcinoma. *Cancer Res.*, 52 (1992) 6774–6781.

Hwang, K. J., Luk, K.K. and Beaumier, P.L., Hepatic uptake and degradation of unilamellar sphingomyelin/cholesterol liposomes: a kinetic study. *Proc. Natl. Acad. USA*, 77 (1980) 4030–4034.

Jain, R.K., Transport of molecules across tumor vasculature. *Cancer Metastasis Rew.*, 6 (1987) 559–593.

Maruyama, K., Yuda, T., Okamoto, A., Kojima, S., Suginaka, A. and Iwatsuru, M., Prolonged circulation time in vivo of large unilamellar liposomes composed of distearoylphosphatidylcholine and cholesterol containing amphipathic poly(ethylene glycol). *Biochim. Biophys. Acta*, 1128 (1992) 44–49.

Mayer, L.D., Tai, L.C.L., Bally, M.B., Mitilenes, G.N., Ginsberg, R.S. and Cullis, P.R., Characterization of liposomal systems containing doxorubicin entrapped in response to pH gradients. *Biochim. Biophys. Acta*, 1025 (1990) 143–151.

Mayer, L.D., Tai, L.C.L., Ko, D.S.C., Masin, D., Ginsberg, R.S., Cullis, P.R. and Bally, M.B., Influence of vesicle size, lipid composition, and drug-to-lipid ratio on the biological activity of liposomal doxorubicn in mice. *Cancer Res.*, 49 (1989) 5922–5930.

Muramatsu, K., Maitani, Y., Machida, Y. and Nagai, T., Effect of soybean-derived sterol and its glucoside mixture on the stability of dipalmitoylphosphatidylcholiine and dipalmitoylphosphatidylcholiine/cholesterol liposomes. *Int. J. Pharm.*, 107 (1994) 1–8.

Olson, F., Mayhew, E., Maslow, D., Rustum, Y. and Szoka. F., Characterization, toxicity and therapeutic efficacy of adriamycin encapsulated in liposomes. *Eur. J. Cancer Clin. Oncol.*, 18 (1982) 167–176.

Qi, X.R., Maitani, Y. and Nagai, T., Effect of soybean-derived sterols on the in vitro stability and the blood circulation of liposomes in mice. *Int. J. Pharm.*, 114 (1995a) 33–41.

Qi, X.R., Maitani, Y. and Nagai, T., Rates of systemic degradation and reticuloendothelial system uptake of calcein in the dipalmitoylphosphatidylcholine liposones with soybean derived sterols in mice. *Pharm. Res.*, 12 (1995b) 48–50.

Qi, X.R., Maitani, Y. and Nagai, T., Relation of rigidity of membrane and stability of dipalmitoylphosphatidylcholine liposomes with soybean-derived sterols prepared by reverse phase evaporation vesicle method. *Chem. Pharm. Bull.*, 44 (1996) 237–240.

Shinozawa, S., Mimaki, Y. and Araki, Y., Determination of the concentration of adriamycin and its metabolites in the serum and tissues of ehrlich carcinoma-bearing mice by high-performance liquid chromatography. *J. Chromatogr.*, 196 (1980) 463–469.

Szoka, F. and Papahadjopoulos, D., Procedure for preparation of liposomes with large internal aqueous space and high capture by reverse-phase evaporation. *Proc. Natl. Acad. Sci. USA*, 75 (1978) 4194–4198.

Unezaki, S., Maruyama, K., Ishida, O., Takahashi, N. and Iwatsuru. M.J., Hanced tumor targeting of doxorubicin by ganglioside GM1-bearing long-circulating liposomes. *J. Drug Targeting*, 1 (1993) 287–292.

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

Woodel, C.M. and Lasic, D.D., Sterically stabilized liposomes. *Biochim. Biophys. Acta*, 1113 (1992) 171–199.