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Enhanced tumor targeting and improved antitumor activity of doxorubicin by long-circulating liposomes containing amphipathic poly(ethylene glycol)

Sakae Unezaki[a], Kazuo Maruyama[b,*], Osamu Ishida[b], Akinori Suginaka[c], Jun-ichi Hosoda[a], Motoharu Iwatsuru[b]

[a]*Department of Pharmacy, Tokyo Medical College Hospital, Nishi-shinjuku, Shinjuku, Tokyo 160, Japan*
[b]*Faculty of Pharmaceutical Sciences, Teikyo University, Sagamiko, Kanagawa 199-01, Japan*
[c]*Research Laboratory, Nippon Oil and Fats Co., Yurakutyo, Chiyoda, Tokyo 100, Japan*

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Abstract

Enhanced delivery of doxorubicin (DXR) to colon 26 in mice was studied by the long-circulating liposomes (LCL) composed of distearoylphosphatidylcholine cholesterol (DSPC CH) (1 1, m m) and 6 mol% distearoyl phosphatidyl ethanolamine (DSPE) derivatives of poly(ethylene glycol) (PEG) with an average molecular weight of 1000. LCL was approximately 100 nm in mean diameter and encapsulated DXR with > 98% entrapping efficiency by the transmembrane pH gradient method. The control liposomes (LP) which had the same lipid composition, similar size and DXR entrapment as LCL, but did not have amphipathic PEG, were used for comparison. Liposomal DXR and free DXR were injected intravenously at a dose of 5 mg DXR kg to Balb c mice implanted subcutaneously with colon 26 carcinoma. DXR encapsulated in LCL showed high blood levels up to 24 h after injection, compared with the corresponding DXR-LP and free DXR. The value of the area under the curve (AUC) of blood was approximately 2.4-or 810-fold higher than that of DXR-LP or free DXR, respectively. LCL decreased the uptake amount of DXR by the reticuloendothelial system (RES) of liver and spleen. Both liposomal formulations effectively reduced the DXR concentrations in heart compared with free DXR. Compared with DXR-LP or free drug, DXR-LCL produced an approximately 3.6-or 10.5-fold increased DXR level in tumor, respectively, at 6 h after injection. The AUC value of tumor tissue for DXR-LCL was 3.4-or 9.4-fold higher than that of DXR-LP or free DXR, respectively. These high tumor levels of DXR by LCL corresponded to the prolonged residence feature of liposomes. Therapeutic experiments were performed with three different formulations of DXR. Administration of DXR-LCL at a dose of 10 mg DXR kg resulted in effective retardation of tumor growth and 2-fold prolongation of survival times compared with DXR-LP, free drug and saline. Our results indicate that DXR encapsulated in long-circulating liposomes is significantly more active against colon 26 carcinoma than the conventional liposome (DSPC CH, 1:1, m m) formulation of DXR and free drug. Thus long-circulating liposomes should be useful carriers of chemotherapeutic agents for the treatment of solid tumor.

Keywords: Liposome; Poly(ethylene glycol); Drug delivery system; Doxorubicin; Colon carcinoma 26

* Tel.:81-426-85-3723, Fax:81-426-85-3432

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1. Introduction

Liposomes have attracted a considerable amount of interest for potential use as a drug delivery system due to their suitable characteristics (Ostro, 1987; Gregoriadis, 1988). Enhancement of therapeutic efficacy and reduction of toxicity of a variety of drugs have been demonstrated with liposome-encapsulated dosage forms. However, the predominant uptake of liposomes by phagocytic cells of the reticuloendothelial system (RES) located mainly in the liver and spleen, and a resulting rapid clearance from the circulation, have been a major obstacle in any attempt to deliver liposomes to cells, tissues or organs other than the RES.

Allen et al. (1987; 1989) have demonstrated that liposomes containing ganglioside GM_1 exhibit a prolonged circulation time in blood by reduction of affinity to the RES cells in liver and spleen. More importantly, this type of small liposome of around 100 nm in mean diameter and rigid lipid composition, such as distearoylphosphatidylcholine/cholesterol (DSPC/CH), showed a higher level of uptake by solid tumors than ordinary liposomes (Gabizon et al., 1988). These reports indicate that liposomes with longer circulation half-lives may have potential applications for drug targeting of solid tumors. Recently, the authors and other groups demonstrated that the inclusion of amphipathic poly(ethylene glycol) (PEG) significantly reduced the RES uptake of liposomes and resulted in effectively prolonged circulation of liposomes (Klibanov et al., 1990; Blume et al., 1990; Senior et al., 1991; Maruyama et al., 1992). We have reported that the inclusion of PEG with a low molecular weight, ranging from 1000 to 2000 on average, within unilamellar large liposomes (200 nm in average diameter) composed of DSPC and CH, resulted in pronounced increase in blood residence time (Maruyama et al., 1992). Amphipathic PEG was more attractive than GM_1, due to it easy synthesis at high levels of purity in large quantities and the resultant considerably lower cost compared to GM_1.

Doxorubicin (DXR) is a potent antineoplastic agent which is active against a wide range of human cancers including lymphomas, leukemia, and solid tumors. However, administration of this drug produces acute toxicity in the form of bone marrow depression, alopecia, and oral ulceration (Olson et al., 1982; Herman et al., 1983; Van Hoesel et al., 1984). The principal dose-limiting toxicity of DXR is cardiotoxicity. A number of investigations in animal systems have shown that DXR entrapped in liposomes reduces non-specific toxicity and maintains or enhances anti-cancer effect. However, there have been few reports on the pharmacokinetic properties and tissue distribution, especially distribution in solid tumors, of DXR encapsulated in small size long-circulating liposomes (LCL) containing amphipathic PEG.

In this paper we investigated the changes in the tissue distribution and pharmacokinetics of DXR when it is delivered by LCL approximately 100 nm in size. In view of the delivery of DXR to the solid tumor, we have investigated the tumor distribution and antitumor efficacy of DXR by LCL in mice bearing colon 26 carcinoma implanted subcutaneously. The chemotherapeutic activities of DXR loaded in LCL, conventional liposomes (LP) and free drug are compared.

2. Materials and method

2.1. Materials

Distearoylphosphatidylcholine (DSPC) (COATSOME MC-8080) and distearoylphosphatidylethanolamine (DSPE) were kindly donated by Nippon Oil and Fats, Tokyo, who also provided monomethoxy poly(ethylene glycol) succinimidyl succinate (PEG-OSu), with average molecular weights of 1000. The number-average molecular weight (M_n), weight-average molecular weight (M_w) and polydispersity (M_w/M_n) of PEG-Osu were measured with gel permeation chromatography. The values of M_n and M_w/M_n were 1047 and 1.08, respectively. Doxorubicin (DXR) was obtained from Kyowa Hakko Kogyo, Tokyo. Cholesterol (CH) was obtained from Wako Pure Chemical, Tokyo. All other chemicals were reagent grade.

2.2. Synthesis of amphipathic PEG

Amphipathic PEG was synthesized systematically by combination of DSPE and PEG-OSu with an average molecular weight of 1000, as described previously by Maruyama et al. (1992). In brief, an aliquot of PEG-OSu in $CHCl_3$ was added to a solution of DSPE in $CHCl_3$/methanol (3:1,v/v), followed by addition of triethylamine to give a 3:1:3.5 molar ratio of PEG-OSu/DSPE/triethylamine. The reaction mixture was stirred overnight at room temperature and organic solvents were evaporated off. Full conversion of the primary amino group in DSPE was confirmed by the negative ninhydrin reactivity after separation of the products by TLC. A small amount of water was added to the evaporated reaction residues to form the micelles. DSPE-PEG micelles were dialyzed against the distilled water using a dialysis bag with large pores (Spectra-Por CE 300000 MWCO, Spectrum Medical) for 5 days and then lyophilized.

2.3. Liposome preparation and DXR encapsulation

LCL was prepared from DSPC/CH (1:1, m/m) and 6 mol% of DSPE-PEG derivative according to the reverse-phase evaporation method (Szoka et al., 1978). The lipid composition of DSPC/CH was used for the preparation of control liposomes (LP) which were used as comparative liposomes in this study. The lipid mixture was dissolved in isopropylether/chloroform (1:1, v/v) and 300 mM citric acid (pH 4.0) were added. The resulting large unilamellar vesicles (LUVs) were extruded ten times through two stacked Nuclepore filters (0.2 and 0.1 μm) to make small size liposomes (SUV). Liposome size was measured by a Nicomp 370 submicron particle analyzer (HIAC Pacific Scientific). DXR encapsulation was done by employing the pH gradient method developed by Mayer et al. (1985, 1989). Briefly, the pH of the liposome suspension, initially at pH 4.0, was raised to pH 7.8 with 1 N NaOH. The liposome preparation was mixed with a preheated (60°C) DXR solution dissolved in distilled water at a drug-to-lipid weight ratio of 0.2. This mixture was incubated with periodic mixing for 10 min at 60°C. The resulting preparation was finally passed through a Sephadex G-50 column. The amount of liposomally entrapped DXR was determined with a fluorescence spectrometer (Hitachi F-3000) by diluting liposomes with 0.3 N HCl–50% ethanol and measuring the fluorescence intensity (E_x, 470 nm; E_m, 590 nm). DXR containing-liposomes were used within 24 h after preparation. There were no change of liposome size and no leakage of the encapsulated drug during the period of storage (4°C).

2.4. Biodistribution of DXR encapsulated in PEG/DSPC/CH liposomes in tumor-bearing mice

Tumor-bearing mice were prepared by inoculating mouse colon 26 carcinoma cells (1×10^5 cells) into the hind foot of Balb/c mice (male, 8 weeks old, and weighting 22–25 g), and the tumor was allowed to grow for approximately 8 days, when the mean of its length and width was 8 mm. The free DXR or liposomal DXR was injected intravenously via the tail vein at a dose of 5 mg DXR/kg. At selected times post injection, blood was collected from the retro-orbital sinus and major organs were excised. Organs were stored at −20°C until assay. A 0.1 g portion of tissue, or whole tissue when it weighed less than 0.1 g, was used for measurement of DXR concentration. Samples were homogenized and extracted with butanol/toluene (1:1, v/v), then the extracts were subjected to HPLC assay according to the method of Masuike et al. (1984).

The HPLC system consisted of a Hitachi 655A-12 high pressure pump, a Hitachi F1000 fluorescence detector (E_x, 470 nm; E_m, 585 nm) and a Nucleosil $_{10}C_{18}$ column (GL Science). The mobile phase was 1 N formic acid–methanol (1:1, v/v) and a flow rate of 1.5 ml/min was used.

2.5. Evaluation of antitumor activity

Tumor-bearing mice were assigned at random into groups of 10. Treatment was started when the tumor had reached a diameter of 8 mm, at approximately 8 days after tumor cell inoculation. The dose per injection was 5 or 10 mg DXR/kg

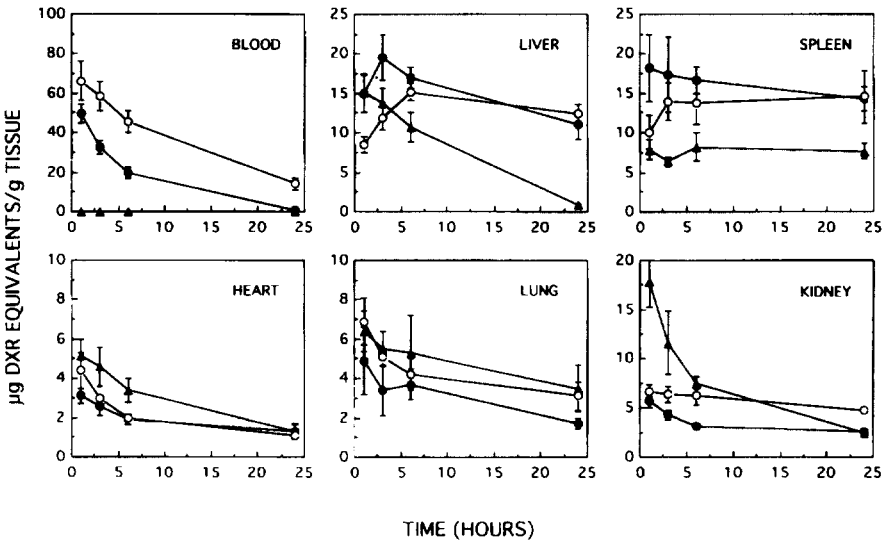

Fig. 1. Drug levels in blood and various tissues after i.v. injection of free DXR (▲), DXR-LP (●) and DXR-LCL (○) into tumor-bearing mice at a 5 mg DXR kg. Results are given as means (S.D.), $n = 3$.

body weight. Treatment was by single i.v. administration via the tail vein. In the efficacy studies, tumor volumes were determined as

$$Volume = L \times W^2 \, 2$$

where L is the longest dimension parallel to the skin surface and W is the dimension perpendicular to L and parallel to the surface (Corbett et al., 1978). Survival times were recorded for a total of 70 days after treatment.

3. Results

3.1. Characterization of liposomal doxorubicin

At 6 mol% of DSPE-PEG, with a mean molecular weight of 2000, in the liposome, there was a little foaming of the liposome preparations. As described by Allen et al. (1991), it was suspected that the foaming was due to the presence of DSPE-PEG micelles which were not incorporated into the liposomes.

The encapsulation of DXR into LCL and LP was done under conditions based on the report of Mayer et al. (1985, 1989), i.e., a pH difference of 3.8 between the inside and outside of the liposome membrane, a DXR lipid weight ratio of 0.2, and a standing time of 10 min at 60°C at the loading step. The mean particle size of all liposome preparations was controlled to within a range of 90–110 nm. Under these fixed conditions, DXR was entrapped into liposomes with approximately 98% efficiency. The presence of DSPE-PEG did not interfere with this procedure. Changes of liposome size were not observed after loading DXR. Furthermore, when liposomes containing PEG were stored at 4°C for long periods, it was observed that the liposome size was constant and the encapsulated drug did not leak (data not shown).

3.2. Biodistribution of DXR encapsulated in liposomes in mice bearing colon 26 carcinoma growing s.c.

Biodistribution of DXR in different formulations was evaluated in tumor-bearing mice until 24 h after injection. Free DXR and liposomal DXR were given to mice via the tail vein at a dose of 5.0 mg DXR/kg. As shown in Fig. 1, free DXR was cleared quickly from blood circulation. At 1 h after injection, a blood concentration of only 0.04

Table 1
Tissue AUC values after i.v. injection of free DXR, DXR-LP and DXR-LCL into the tumor-bearing mice at a dose of 5 mg DXR/kg

Formulation	Tissue AUC[a] (h·μg/g)						
	Blood[b]	Liver	Heart	Lung	Spleen	Kidney	Tumor
Free DXR	1.0	168.8	63.2	106.4	178.1	146.1	18.1
DXR-LP	342.8	341.4	41.2	67.5	365.5	72.4	50.1
DXR-LCL	809.5	309.4	41.9	91.4	320.3	132.0	169.6

[a]AUC values are calculated for 1–24 h.
[b]Blood AUC value is given as h·μg/ml.

μg/ml was observed. In contrast, the blood level of DXR encapsulated in liposomes remained high for a long period. In particular, the blood concentration of DXR-LCL was higher than that of DXR-LP. As summarized in Table 1, the area under the concentration-time curve (AUC) of DXR-LCL was 2.4-or 810-times higher than that of DXR-LP or free DXR, respectively. The drug level of DXR-LCL in the liver and spleen was significantly diminished as compared with DXR-LP within 6 h after injection. Thus, the incorporation of PEG into liposomes resulted in an increased blood level of DXR and decreased drug levels in RES. In the heart, the drug concentration was lower with both liposome formulations compared to free DXR. Similar results were obtained in the lung. AUC values of heart and lung for DXR-LCL were lower than that of free DXR (Table 1). The drug levels in the kidney was markedly different between free DXR and liposomal DXR. DXR encapsulated in LCL maintained same level up to 24 h post-injection. This result indicated that LCL was stable, and entrapped DXR was gradually released from LCL in the circulation.

Fig. 2 shows the DXR levels in solid tumor after administration of free DXR and liposomal DXR at a dose of 5.0 mg DXR/kg. The tumor concentration of free DXR at 1 h after injection was 1.1 μg/g, and did not increase thereafter. Administration of DXR-LP produced increased accumulation of DXR in solid tumor compared with free drug, and resulted in a tumor concentration of 3.5 μg/g at 3 h after injection. The highest drug levels in tumor were obtained by the administration of DXR-LCL at 6 h after injection. The DXR concentration was 10.5 μg/g, and was 3.6-fold higher than that of DXR-LP. The AUC values of tumor (Table 1) for DXR-LCL and DXR-LP were 9.4- and 2.8-fold greater than that of free DXR, respectively. It is clear from Figs. 1 and 2 that such high tumor accumulation of DXR directly correspond to the continued high blood concentration of long-circulating liposomes.

3.3. Antitumor activity of DXR encapsulated in liposomes

The ratios of tumor growth on days 7 and 14 after treatments are shown in Table 2. The tumor growth in DXR-LCL was delayed for a longer

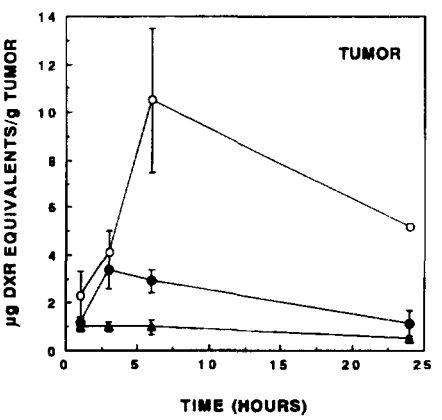

Fig. 2. Drug levels in tumor after i.v. injection of free DXR (▲), DXR-LP (●) and DXR-LCL (○), into tumor-bearing mice at a dose of 5 mg DXR/kg. Results are given as means (S.D.), $n = 3$.

Table 2
Effects of free DXR, DXR-LP and DXR-LCL on tumor growth and survival of mice bearing colon carcinoma 26 tumor

Treatment	Dose (mg/kg)	Tumor growth ratio[a]		Survival time		%ILS[b]
		day 7	day 14	mean	median	
Control		12.3 (2.8)	27.7 (6.0)	22.2	22	–
Free DXR	5	6.6 (1.6)	15.6 (1.6)	24.2	24	9
	10	5.5 (1.5)	10.6 (1.9)	20.6	21	−7
DXR-LP	5	7.5 (1.0)	16.1 (2.8)	24.7	23.5	11
	10	5.7 (0.8)	9.8 (1.6)	27.0	26	21
DXR-LCL	5	3.8 (0.9)	9.0 (2.2)	31.0	30	40
	10	1.5 (0.8)	2.6 (1.6)[c]	47.6	46	114

[a]Tumor growth ratio, mean tumor volume at 7 or 14 days after treatment/mean tumor volume at day of treatment (day 0). Data are the means (S.D.) of 10 mice per group.
[b]Increase in life span (ILS), (mean survival time (MST) treated mice/MST controls × 100) − 100.
[c]$P < 0.01$, compared to other treatments.

time than that in the other treatments. The delay was dependent on the DXR dose. Fig. 3 shows the survival curves of mice inoculated with mouse colon carcinoma 26 cells and treated i.v. with free DXR and liposomal DXR at doses of 5 or 10 mg DXR/kg. There was no improvement in survival time for the administration of DXR-LP and free DXR. In contrast, the administration of DXR-LCL had a tendency to prolong the survival time at a dose of 5 mg DXR/kg, and showed significantly increased survival time at a dose of 10 mg DXR/kg. The median survival time of DXR-LCL (10 mg DXR/kg) administration was 46 days which was greater than that of other treatment (Table 2). DXR-LCL produced 2-fold increase in survival time compared with DXR-LP and free DXR. These results show clearly that DXR encapsulated in long-circulating liposomes causes a marked improvement in therapeutic efficacy, inhibiting tumor growth.

4. Discussion

Many reports in various animal models have demonstrated that liposomal entrapment of DXR can significantly reduce the acute and chronic toxic side effects associated with use of free DXR (Rhaman et al., 1980; Olson et al., 1982; Gabizon et al., 1986). However, conventional liposome formulations of DXR are rapidly removed from the circulation by the phagocytic cells of the RES resulting in reduction of the amount of the drug that will reach non-RES sites, including solid tumors. To overcome these problems in drug delivery to the tumor site, liposomes with long circulation time are required. Allen and Chonn (1987) have demonstrated that GM_1-containing liposomes composed of rigid lipids and cholesterol exhibited a prolonged circulation time in blood and a reduced RES uptake. Gabizon and Papahadjopoulos (1988) also demonstrated that GM_1-containing liposomes with 100 nm in size could be accumulated into implanted tumors at a high rate compared with conventional liposomes. In the present study, we have obtained evidence on the selective tumor accumulation and superior therapeutic efficacy of DXR encapsulated in LCL with, on average, a diameter of 100 nm in a colon 26 tumor model. Although the mechanism of effect of PEG in prolonging the circulation time of liposomes has not been fully clarified, an increased hydrophilicity (Senior et al., 1991) and /or steric barrier on the liposomal surface (Klibanov et al., 1991; Mori et al., 1991) might be provided by amphipathic PEG. This hydrophilic surface in turn may prevent or reduce the interactions of liposomes with serum constituents, resulting in an enhanced stability of liposomes and a reduced rate of RES interaction. Thus, PEG-containing liposomes are considerably advantageous for clinical and pharmaceutical applications, stand a bet-

ter chance of penetrating the leaky vasculature of solid tumor and may release their contents slowly over a long period in circulating blood. The present results clearly indicate that the encapsulation of DXR into liposomes with prolonged circulation time is an important requirement for the enhanced accumulation of DXR in tumor tissue.

The mechanism of liposome accumulation in solid tumors is not fully clear at present. Normal tissues outside the RES are known to generally have continuous and nonfenestrated vascular endothelia, and extravasation of macromolecules such as liposome is greatly limited. On the other hand, the permeability of the endothelial barrier in newly vascularized tumors is increased compared to normal tissues (Jain et al., 1986). Under physiological tumor conditions, it is possible that small liposomes (less than 100 nm) with prolonged circulation half-lives could predominantly pass through leaky endothelium into tumor areas by passive convective transport. However, penetration into the tumor cells is not expected for particles of the size of liposomes. It is possible to speculate that intact extravasated liposomes within the tumor area gradually break down and release entrapped drug. Amphipathic molecules such as DXR, once released from the liposomes, would then be able to quickly penetrate deep into the tumor mass. In the present study, the prolonged survival times and the greater tumor growth inhibition indicate that DXR encapsulated in LCL remains to be bioavailable and active against colon 26 tumor in mice.

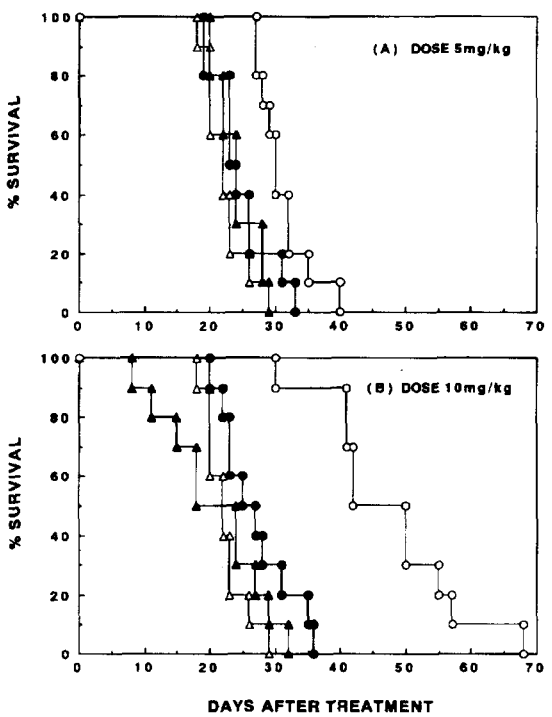

Fig. 3. Colon 26 antitumor activity after single i.v. injection of 5 (A) or 10 (B) mg DXR/kg free doxorubicin and liposomal doxorubicin. Survival curves were derived from groups of 10 BALB/c mice inoculated s.c. with 1×10^5 colon 26 cells and shown for control (△), free DXR (▲), DXR-LP (●), and DXR-LCL (○).

As shown by Mayer et al. (1989), the encapsulation of DXR in DSPC/CH liposomes causes a dramatic increase in the LD_{50}, and a high DXR-to-lipid ratio decreases the acute toxicity of liposomal DXR. In this experiment, 98% trapping efficiency was obtained for a drug-to-lipid weight ratio of 0.2:1 by pH gradient method. The present data showed that with both types of liposomal formulations the drug concentration in heart was lower than with free DXR, therefore the resulting time of exposure by DXR was decreased compared with that of free DXR. It was clear that this behavior was related to the distribution of liposomes. Although the released drug and liposome-encapsulated drug fraction in the plasma were not assayed, it is safe to assume that the drug concentration is associated with the circulating liposomes due to the rapid clearance of free DXR.

The novel liposomes with prolonged circulation time selectively distribute encapsulated drug directly to tumor sites. Furthermore, our present data indicate that DXR encapsulated in PEG-liposomes yields superior therapeutic efficacy than free DXR at equivalent doses.

In conclusion, long-circulating liposomes, as drug carriers, yield potential applications in cancer chemotherapy, with reduction of toxicity and selective drug delivery. The enhanced therapeutic efficacy of DXR encapsulated in PEG-liposomes is probably related to the extended circulation time of the formulation and its accumulation in tumor. DXR encapsulated in long-circulating liposomes should be more useful for cancer

chemotherapy than the former liposomal DXR and free DXR.

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