Full Paper

A Pegylated Liposomal Platform: Pharmacokinetics, Pharmacodynamics, and Toxicity in Mice Using Doxorubicin as a Model Drug

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Abstract. Aims were to observe pharmacokinetics, pharmacodynamics, and toxicity for constructing a Sino-pegylated liposomal platform. Human hepatocarcinoma cells (Bel7402) and murine hepatocarcinoma cells (H₂₂) were used for the cytotoxicity assay and the in vivo solid xenograft tumor model in mice, respectively. Pharmacokinetic results in mice showed that the pegylated liposomal doxorubicin markedly prolonged the blood circulation of doxorubicin. Elimination half-time ($T_{1/2,\gamma}$) of pegylated, regular liposomal doxorubicin and free doxorubicin were 46.09 ± 14.44 , 26.04 ± 3.34 , and 23.72 ± 5.13 h, respectively. The area under the concentration-time curves ($AUC_{0-\infty}$) ($h \cdot \mu g/g$) of the pegylated and regular liposomal doxorubicin were 6.8- and 2.6-fold higher than that of free doxorubicin, respectively. Cytotoxicity and antitumor activity in vivo indicated that activity of the pegylated liposomal doxorubicin was higher than that of the regular or the free one, respectively. After two weeks of tail intravenous injection of the pegylated liposomal doxorubicin at a single dose of 10 mg/kg, no significant damage was observed in gastric, intestinal mucosa, and heart muscle, but pronounced damages were found in the control group after dosing free doxorubicin. The results demonstrate that the pegylated liposomes improve the efficacy of toxics and reduce the toxicity, therefore providing favorable evidence for building a pegylated liposomal platform.

Keywords: pegylated liposomal platform, doxorubicin, pharmacokinetics, pharmacodynamics, toxicity

Introduction

Liposomes were initially defined in the 1960s, but only in the early 1990s did the first therapeutic liposomes appear on the market. Although the coverage is slightly different, the “generations” of liposomes are described by Cattell et al. (1) as four generations, namely, 1) conventional liposomes like the antifungal drug amphotericin B (Ambisome, by Nexstar, Boulder, CO, USA) and doxorubicin for antimetastatic breast cancer (Myocet, by Elan Pharma Int., Princeton, NJ, USA); 2) long-circulating liposomes like daunorubicin liposomes (Daunosome) using “pure lipids” as excipient materials for treating AIDS-related Kaposi’s sarcoma; 3) modified liposomes using gangliosides or sialic acid

for evading the immune system rapid uptake, thus achieving long-circulation of drugs in blood; 4) pegylated liposomal doxorubicin, also being called “stealth liposomes”, (Doxil, by Schering-Plough, Madison, NJ, USA) for curing AIDS-related Kaposi’s sarcoma, resistant ovarian cancer, and metastatic breast cancer. Because from the second to the fourth generation, the objective is to achieve long-circulation in blood, long-circulation liposomes may be described as “the second generation” even though the liposome materials are different. The fifth generation of liposomes may be the more targeting ones like immunoliposomes, which combine both stealth and active targeting properties (e.g., conjugating monoclonal antibody) together. However, the specific monoclonal antibodies to tumor tissues are not easily available because most of the overexpressed antigens in tumors are also expressed in normal tissues, and few antigens specifically expressed

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only in tumors have been identified. The pegylated liposomes have a very long-circulation half-life, much lower toxicity in comparison with free drug, and accumulate more in tumors through leaky vessels, thus enhancing the efficacy (2). A pegylated liposomal platform is potentially useful and is intended to encapsulate serial drugs. The mechanism accounting for the long-circulation of the pegylated liposomes is supposed to be that the bulky polyethylene glycol (PEG) head group served as a barrier preventing interactions with plasma opsonins as a result of its concentration of highly hydrated groups that sterically inhibit hydrophobic and electrostatic interactions of a variety of blood components at the liposome surface, as described by Ceh et al. (3). The main questions for construction of such a platform are how to get high encapsulation efficiency for many drugs and obtaining direct evidence showing that pegylated liposomes lower the toxicity.

Doxorubicin, a potent antineoplastic agent against a wide range of human tumors, is used alone and in combination with other drugs for treatment of tumors including malignant lymphomas and leukemias. However, injection of doxorubicin hydrochloride (free drug) is associated with severe acute heart and gastrointestinal toxicity. Other side effects include possible hair loss, mouth inflammation, and so on. Doxorubicin is a useful drug for construction of such a pegylated liposome platform. High encapsulation efficiency (>90%) has been achieved for doxorubicin and for some other drugs by chemical gradients (3–5), for example, ammonia sulfate gradients and pH gradients, or the divalent calcium cation ionophore A23187 method (6). Many studies (7) have investigated the tissue distribution of doxorubicin after injection of pegylated liposomal doxorubicin in rodents with syngeneic or xenogeic tumor models but the detection is fluorescence-based, and doxorubicin fluorescence is partially quenched when doxorubicin binds DNA (8). Our investigation applied HPLC-UV method for detection of doxorubicin. The aims of present study were to observe pharmacokinetics and pharmacodynamics and direct evidence for lowering toxicity in vivo of pegylated liposomes using mice.

Materials and Methods

Pegylated doxorubicin liposomes

Pegylated doxorubicin liposomes were made according to Haran et al. (5). Briefly, egg phosphatidylcholine (EPC), cholesterol, and polyethylene glycol-distearoylphosphosphatidylethanolamine (PEG₂₀₀₀-DSPE; NOF Corporation, Tokyo) (55/40/5, $\mu\text{mol}/\mu\text{mol}/\mu\text{mol}$) and 2% (w/w) vitamin E were dissolved in chloroform in a

pear-shaped flask. The chloroform was evaporated to dryness under vacuum with a rotary evaporator, and the lipid film was then hydrated with 120 mM ammonium sulfate by sonication in the water bath at 60–65°C for 10 min, and then blank pegylated liposomes were made and were successively extruded through polycarbonate membranes (Nuclepore, Pleasanton, CA, USA) with pore size of 600, 400, and 200 nm for 3 times, respectively. The mean diameter of blank pegylated liposomes was about 100 nm. The doxorubicin liposomes were produced by the following procedure: After dialysis in the solution of 5% glucose for 4 times, blank pegylated liposomes were mixed with doxorubicin hydrochloride (Shantou Tobing Pharmaceutical Factory, Shantou, China) dissolved in phosphate buffer (pH 6.8) in a 60°C water bath and intermittently shaken in the water bath at 60°C for 30 min. The regular doxorubicin liposomes were prepared by the same procedure excluding the addition of PEG-DSPE in the formed lipid membrane. Both pegylated doxorubicin liposomes and regular liposomes had high entrapment efficiency (>95%).

Pharmacokinetics

Male Kun-Ming mice (body weight 20 ± 2 g, 5-week-old, obtained from Experimental Animal Center of Peking University Medical Center) were equally divided into 6 groups (6 mice for each group) and housed under conditions of $25 \pm 3^\circ\text{C}$ and 50% humidity, with free access to standard food and water. Pegylated doxorubicin liposomes, regular doxorubicin liposomes, or doxorubicin hydrochloride injection were given to mice via a tail vein at a single dose of 5 mg/kg. At 0, 0.25, 0.5, 1, 3, 5, 24, and 48 h, the mice were sacrificed; and the blood, heart, liver, kidney, stomach, and small intestine were immediately collected. The remaining blood in the heart, liver, kidney, stomach, and small intestine tissues was carefully removed by washing with distilled water so that the contribution of the blood to the data of each tissue was minimal. Mice for the control group were injected with biological saline. The bloods and tissues were homogenized in 0.8 ml of citric acid buffer (pH 6) for 1–3 min at a speed of 3000 rpm. The homogenate was transferred to a centrifugation tube, then 3.0 ml of mixture solution of chloroform and methanol (1/4, v/v) was added to the same tube and shaken using a vortex mixer for 1 min, and centrifuged at 2500 rpm for 15 min. After centrifugation, 3.0 ml of the chloroform layer was transferred to another tube and evaporated to dryness under nitrogen gas in the bath at 50°C. The residues were dissolved in the 200 μl of methanol and injected into an HPLC system (TSP, Muskegon, MI, USA). The detection wavelength was 254 nm, and the mobile phase was

acetonitrile-water (32/68, v/v, pH adjusted to 3.0 by phosphoric acid). The flow rate was 1.5 ml/min, and the analytical column was of C₁₈ (4.6 × 250 mm, 5 μm). The extraction efficiency was more than 89%, and the mean relative standard deviation was less than 10% in the intra-day and inter-day determination. The detection limit was 50 ng/g tissue.

The pharmacokinetic parameters were calculated from the average blood concentrations by the pharmacokinetic computer program PKBP-N1 (China). The same sampling-point concentrations of pegylated or regular liposomal doxorubicin *versus* those of free doxorubicin were statistically analyzed using the analysis of variance (ANOVA), respectively.

In vitro and in vivo pharmacodynamics

Human hepatocarcinoma cell (Bel7402) and murine hepatocarcinoma cell (H₂₂) lines were gifts from Prof. Cui Jing-Rong (Department of Molecular Pharmacology, Peking University, China).

Sulforhodamine B (SRB) assay

Human hepatocarcinoma cell (Bel7402) cytotoxicity was determined using the SRB colorimetric anticancer-drug screening assay (9). Briefly, pegylated-liposomal, regular liposomal, and free doxorubicin were each diluted using biological saline to the following concentrations: 0.1, 0.5, 1.0, 5.0, 10.0, and 50.0 μg/ml. Biological saline was used as a blank control. Appropriate amounts of cells (1.5 × 10⁴/ml) were seeded onto 96-well flat-bottomed plates in 180 μl of RPMI 1640 medium (containing doxorubicin or biological saline). After incubation for 0, 24, 48, and 72 h, cells in the 96-well plates were washed with water, fixed with 10% ice-cold trichloroacetic acid at 4°C for 1 h, then washed with water five times, and dried at 60°C. The cellular proteins in each well were stained with 100 μl of 0.4% SRB in 1% acetic acid at room temperature for 20 min, then washed with 1% acetic acid four times, and dried at 37°C for another 30 min. To dissolve the SRB bound to cellular protein, 200 μl of 10 mM Tris were added to each well and incubated at room temperature with mechanical agitation until the color became homogeneous. SRB bound to protein was measured by absorbance at a 540-nm wavelength using a Micro-Plate Reader (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

Xenograft solid tumor model in mice

Male mice were divided into 12 groups, weighed (20 ± 2 g), and labeled; each group consisted of 10 mice. Murine hepatocarcinoma cells (5 × 10⁶/ml, H₂₂) were inoculated via the subcutaneous tissue of mouse right limb armpit (0.2 ml for each mouse). Pegylated

liposomal doxorubicin, regular liposomal doxorubicin, and doxorubicin hydrochloride (free type) were each administered at a single dose of 5 or 10 mg/kg on the 2nd or 7th days after tumor inoculation. The animals were killed on the 15th day after tumor inoculation. The tumor was removed and weighed.

Gastrointestinal and cardio-toxicity

Male mice were divided into 6 groups, each group consisted of 10 mice and conditions were the same as those under the above pharmacokinetic study. Pegylated liposomal doxorubicin (10 or 15 mg/kg, a single dose), regular liposomal doxorubicin (10 or 15 mg/kg, a single dose), or doxorubicin hydrochloride injection (10 mg/kg, a single dose) were given via a tail vein. After two weeks, the mice were sacrificed; and specimens including heart, stomach, and upper section of small intestine were collected, washed, fixed using 10% formaldehyde, and then imbedded using paraffin and sliced and dyed using hematoxylin and eosin (HE). Pictures for pathological slices were taken under a microscope.

Results

Pharmacokinetics

The profile of doxorubicin in the blood after tail intravenous injection of the 5 mg/kg dose of pegylated doxorubicin liposomes, regular doxorubicin liposomes, and doxorubicin hydrochloride solution are shown in Fig. 1A. The doxorubicin levels in blood for the three groups of mice decreased sharply with time. The blood concentrations of pegylated liposomal doxorubicin were pronouncedly higher than those of regular liposomal doxorubicin ($P < 0.01$) 1 h after administration or those of free doxorubicin ($P < 0.001$) 0.5 h after administration. According to AIC values (Akaike's Information Criterion), the blood data were fitted with a three-compartmental model. The area under the concentration-time curves (AUC_{0-∞}) was 499.61 ± 72.35 h·μg/g for pegylated liposomal doxorubicin, 188.73 ± 88.10 h·μg/g for regular liposomal doxorubicin, and 72.98 ± 15.79 h·μg/g for free doxorubicin. Elimination half-life ($T_{1/2, \gamma}$) was 46.09 ± 14.44 h for the pegylated, 26.04 ± 3.34 h for the regular, and 23.72 ± 5.13 h for free doxorubicin, as indicated in Table 1. Results of pegylated liposomal groups showed that pegylated carriers increased the exposure of doxorubicin in the blood, and prolonged the half-life of doxorubicin in comparison with those of the regular or free doxorubicin.

The concentrations of doxorubicin in the heart, liver, kidney, stomach, and small intestine after administration

A. Doxorubicin in mice bloods

B. Doxorubicin in mice heart tissues

C. Doxorubicin in mice liver tissues

D. Doxorubicin in mice kidney tissues

E. Doxorubicin in mice stomach tissues

F. Doxorubicin in mice small intestine tissues

Fig. 1. Doxorubicin concentrations after tail intravenous injection of pegylated liposomal doxorubicin, regular liposomal doxorubicin, and free doxorubicin at a single dose of 5 mg/kg. Each line or column shows the mean $\pm$ S.E.M. ($n = 6$ for each). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, vs free doxorubicin at the same sampling time-point, respectively; $\Delta\Delta P < 0.01$, vs regular liposomal doxorubicin.

Table 1. Pharmacokinetic parameters using three-compartmental model in mice following tail intravenous injection of pegylated liposomal, regular liposomal, and free doxorubicin at a single dose of 5 mg/kg

Parameters	Pegylated liposomal doxorubicin	Regular liposomal doxorubicin	Free doxorubicin
α (h ⁻¹)	4.518 ± 3.961	0.995 ± 0.031	4.904 ± 7.458
T _{1/2,α} (h)	0.35 ± 0.37 (0.08 – 1.06)	0.70 ± 0.02 (0.68 – 0.74)	0.35 ± 0.21 (0.03 – 0.68)
β (h ⁻¹)	0.286 ± 0.078	0.789 ± 0.189	0.202 ± 0.207
T _{1/2,β} (h)	2.56 ± 0.63 (1.66 – 3.33)	0.93 ± 0.26 (0.69 – 1.34)	6.34 ± 4.02 (1.17 – 11.95)
γ (h ⁻¹)	0.016 ± 0.004	0.027 ± 0.003	0.031 ± 0.007
t _{1/2,γ} (h)	46.09 ± 14.44***,ΔΔ (34.65 – 69.30)	26.04 ± 3.34 (22.35 – 30.13)	23.72 ± 5.13 (16.90 – 28.88)
V _{ss} (L)	0.58 ± 0.16	0.81 ± 0.20	1.42 ± 0.45
AUC _{0–∞} (h · μg/g)	499.61 ± 72.35***,ΔΔ	188.73 ± 88.10	72.98 ± 15.79
C _{max} (μg/g)	35.19 ± 1.85	36.21 ± 3.02	28.96 ± 7.41

*** $P < 0.01$, *** $P < 0.001$, vs free doxorubicin; ΔΔ $P < 0.01$, vs regular liposomal doxorubicin.

of the 5 mg/kg dose of pegylated liposomal doxorubicin, regular liposomal doxorubicin, and free doxorubicin are described in Fig. 1B, C, D, E, and F, respectively. 1) Concentrations of pegylated or regular liposomal doxorubicin in heart tissues were significantly lower at 1 h ($P < 0.001$), at 3 h ($P < 0.001$), and at 5 h ($P < 0.05$) than those of free doxorubicin at the corresponding time point, respectively. 2) Concentrations of pegylated or regular liposomal doxorubicin in liver tissues were slightly higher at 1 h, significantly higher at 3 h ($P < 0.01$), and higher at 5 h (without significance) than those of free doxorubicin at the corresponding time point, respectively. 3) Concentrations of pegylated or regular liposomal doxorubicin in kidney tissues were significantly lower at 1 h ($P < 0.01$), at 3 h ($P < 0.05$), and at 5 h ($P < 0.05$) than those of free doxorubicin at the corresponding time point, respectively. 4) Concentrations of pegylated or regular liposomal doxorubicin in stomach tissues were significantly lower at 1 h ($P < 0.05$), at 3 h ($P < 0.01$), and at 5 h ($P < 0.05$) than those of free doxorubicin at the corresponding time point, respectively. 5) Concentrations of pegylated or regular liposomal doxorubicin in small intestinal tissues were significantly lower at 1 h ($P < 0.01$), at 3 h ($P < 0.05$), and at 5 h ($P < 0.001$) than those of free doxorubicin at the corresponding time point, respectively.

Pharmacodynamics

In vitro inhibition rates (%) to human hepatocarcinoma cells are shown in Table 2, depicting that the antitumor activities for both liposomal groups were similar ($P > 0.05$) and were markedly higher ($P < 0.05$) than that of free doxorubicin groups at a low dose of

administration (doxorubicin concentration ≤ 1.0 μg/ml). At a higher dose of 5.0 or 50.0 μg/ml of doxorubicin, the inhibition rates for the three groups were close at different time points.

In vivo inhibition rates (%) of solid tumor weight are shown in Fig. 2. In the groups following a single dose of 5 mg/kg, the antitumor activity of pegylated liposomal doxorubicin either after dosing on the 2nd day or dosing on the 7th day was higher ($P < 0.05$) than that of regular liposomal doxorubicin after dosing on the same day. Similar situations were found in the groups following a single dose of 10 mg/kg, and the antitumor activity of the pegylated liposomal doxorubicin was higher ($P < 0.05$) after dosing on the 2nd day or slightly higher after dosing on the 7th day than that of regular liposomal doxorubicin after dosing on the same day, respectively. In comparison with free doxorubicin, the inhibition rate (%) for both liposomal doxorubicins were higher ($P < 0.05$) than that of free doxorubicin. A comparison of activity between the 2nd day and 7th day dosing scheme showed that the antitumor activities of 7th day dosing groups were slightly higher than those of the 2nd day dosing groups.

Toxicity

Pathological slices of gastric mucosa showed that the gastric gland dilation and cell dropping along with topical inflammatory infiltration were found in the free doxorubicin groups (10 mg/kg), as depicted in Fig. 3A. No damages in the stomach were found in pegylated (as shown in Fig. 3B) and regular liposomal groups at a single dose of 10 mg/kg in comparison with normal gastric mucosa of mice.

Table 2. Inhibition rate (%) of pegylated liposomal doxorubicin, regular liposomal doxorubicin, and free doxorubicin to growth of human hepatocarcinoma cells (Bel7402)

Concentration ($\mu\text{g/ml}$)	0.01	0.10	0.50	1.00	5.00	50.00
Pegylated						
24 h	8.09 ± 2.31	7.83 ± 1.62	$26.89 \pm 3.73^*$	$32.38 \pm 2.64^*$	$54.05 \pm 1.50^*$	56.66 ± 6.10
48 h	$38.15 \pm 5.10^*$	$36.57 \pm 3.61^*$	$67.04 \pm 2.86^{**}$	$74.27 \pm 3.66^{**}$	$89.16 \pm 3.18^{**}$	96.84 ± 3.87
72 h	$50.73 \pm 3.87^*$	58.29 ± 5.50	$74.50 \pm 6.10^*$	$88.71 \pm 4.16^*$	97.09 ± 2.39	96.54 ± 2.91
Regular						
24 h	1.83 ± 0.21	$17.49 \pm 1.03^*$	$24.54 \pm 2.60^*$	$31.85 \pm 3.30^*$	$44.13 \pm 3.30^*$	59.27 ± 3.31
48 h	26.19 ± 0.21	$34.09 \pm 2.80^*$	$62.75 \pm 3.94^{**}$	$67.27 \pm 3.07^{**}$	$91.65 \pm 5.13^{**}$	93.91 ± 6.13
72 h	$51.37 \pm 4.60^*$	54.28 ± 3.12	$85.61 \pm 2.76^*$	$96.72 \pm 2.86^*$	97.63 ± 3.00	94.17 ± 5.01
Free						
24 h	11.75 ± 2.11	9.66 ± 3.20	16.19 ± 1.23	16.97 ± 3.76	30.55 ± 2.76	69.19 ± 3.36
48 h	25.28 ± 3.32	21.15 ± 2.81	23.76 ± 2.90	31.33 ± 3.62	57.18 ± 2.89	95.82 ± 3.72
72 h	22.04 ± 2.89	54.83 ± 6.70	49.36 ± 3.56	54.83 ± 7.70	86.15 ± 6.25	97.45 ± 3.11

* $P < 0.05$, ** $P < 0.01$, vs free doxorubicin.

Fig. 2. Inhibition rate (%) of solid tumor weight after inoculation of murine hepatocarcinoma cell (H_{22}) and subsequent treatment with the pegylated, regular liposomal, and free doxorubicin on the 2nd day and on the 7th day (either 5 or 10 mg/kg). The antitumor activity of the pegylated liposomal doxorubicin was higher than that of the regular one. Each column shows the mean $\pm$ S.E.M. ($n = 10$ for each). * $P < 0.05$, vs free doxorubicin; $^{\Delta}P < 0.05$, vs regular liposomal doxorubicin.

Denaturations of gland particles and Paneth's cells (Fig. 4A) in the mucosa of the upper small intestine were observed in the free doxorubicin groups (10 mg/kg). No pronounced damage was found in the two liposomal groups at a single dose of 10 or 15 mg/kg, compared to the blank control group. The normal slice picture for pegylated liposomal doxorubicin (15 mg/kg)

is shown in Fig. 4B, indicating that the pegylated liposomal doxorubicin significantly reduced the intestinal toxicity.

Eosinophilic-like plasma myocytes before necrosis in the heart muscle of mice were observed after two weeks of injection of free doxorubicin (10 mg/kg). In addition, fatty denaturation and fatty degeneration of papillomuscle (Fig. 5A) were found in this group, compared to the pegylated liposomal doxorubicin (10 mg/kg) group (Fig. 5B). On the contrary, no damage in heart muscle was found in two liposomal groups at a single dose of 10 mg/kg, but the slight fatty denaturation in heart muscle was found at a single dose of 15 mg/kg.

Discussion

In the preparation of liposomes, both pegylated liposomal and regular liposomal doxorubicin were produced by the ammonium sulfate gradient method. In the preparation of regular liposomes, only PEG-DSPE was not added. The liposome suspension was stored in a 4°C refrigerator for 10 months after filling nitrogen gas into the vials of liposomes and no pronounced sediment was found. EPC and hydrogenated soybean phosphatidylcholine (HSPC) were separately used for preparation of liposomes; the results of present investigations were from the liposomes using EPC, which differed from that of Doxil using HSPC (5). Antioxidant vitamin E was used in both formulations, and it seemed that both stabilities of liposomes using EPC and the one using HSPC were not markedly different according to the results of stability (data not shown).

Concentrations of doxorubicin and its metabolites in

Fig. 3. Pathological slice micrographs of gastric mucosa following two weeks of tail intravenous injection of free doxorubicin or pegylated liposomal doxorubicin at a single dose of 10 mg/kg (10×40 , HE dye), respectively. A: Cell dropping of the mouse gastric gland after i.v. administration of free doxorubicin. B: Normal gastric mucosa of mice after i.v. administration of pegylated liposomal doxorubicin. Results showed that the free doxorubicin caused severe damage in mouse gastric mucosa. In contrast, no significant injury in mouse gastric mucosa was observed after administration of pegylated liposomal doxorubicin.

Fig. 4. Pathological slice micrographs of small intestine mucosa following two weeks of tail intravenous injection of free doxorubicin at a single dose of 10 mg/kg or pegylated liposomal doxorubicin at a single dose of 15 mg/kg (10×20 , HE dye), respectively. A: Paneth's cell degeneration in the small intestine after i.v. administration of free doxorubicin. B: Normal mucosa in the small intestine of mice after i.v. administration of pegylated liposomal doxorubicin. Results showed that the free doxorubicin caused severe damage in mouse small intestine mucosa. In contrast, no pronounced injury in mouse small intestine mucosa was observed after administration of pegylated liposomal doxorubicin at a higher dose.

Fig. 5. Pathological slice micrographs of heart muscle following two weeks of tail intravenous injection of free doxorubicin or pegylated liposomal doxorubicin at a single dose of 10 mg/kg (10×20 , HE dye), respectively. A: Fatty denaturation in the heart muscle of mice after i.v. injection of free doxorubicin. B: Normal heart muscle of mice after i.v. injection of pegylated liposomal doxorubicin. Results showed that the free doxorubicin caused severe damage in mouse heart muscle. In contrast, no pronounced injury in mouse heart muscle was observed after administration of pegylated liposomal doxorubicin.

the serum and tissues have been determined by the HPLC-fluorescence method (10–13). According to MEDLINE, only one paper has used the HPLC-UV method for determination of doxorubicin in biological samples for pharmacokinetic and pharmacodynamic studies used in nasopharyngeal carcinoma patients (14). We modified and developed a simple HPLC-ultraviolet method for separation of doxorubicin from biological materials. The recovery and precision studies showed that the established method met the requirement of biological samples.

The studies of pharmacokinetics in mice showed that both pegylated liposomal and regular liposomal doxorubicin significantly changed transport and distribution of doxorubicin in the heart, liver, kidney, stomach, and small intestine tissues of mice. In blood, doxorubicin displayed multiphase curves with a rapid decline of the initial blood concentration. The first phases of the three formulations showed rapid distributions with half-lives from 0.03 to 1.06 h, but the half-lives in the second phases of two liposomal doxorubicins were significantly shorter than those of free doxorubicins (Table 1). The third phases were the elimination phase with an extended half-life on the average of 46.09 h for the pegylated liposomal doxorubicin in comparison with that of 26.04 h for regular doxorubicin or with that of 23.72 h for free doxorubicin, demonstrating that the pegylated liposomal doxorubicin pronouncedly prolonged the circulation time. The reported half-life of elimination in rats and dogs for Doxil, the pegylated liposomal doxorubicin, were 35 (15) and 27 h (16), respectively. The $AUC_{0-\infty}$ ($h \cdot \mu g/g$) for the pegylated and for the regular compound were 6.8- and 2.6-fold higher than that of free doxorubicin, showing that the liposomal doxorubicin exposure in the blood circulation was significantly increased in comparison with free doxorubicin, and the pegylated liposomal doxorubicin was more favorable for the long-circulation.

In the heart, kidney, stomach, and small intestinal tissues, both liposomes reduced the concentrations of doxorubicin following tail intravenous injection in comparison with those of free doxorubicin, thus indicating that the lower concentration of doxorubicin in these tissues may lead to a lower toxicity of doxorubicin. However, the two liposomal doxorubicins in liver tissues seemed to be higher within 24 h in comparison with free drug, although doxorubicin concentrations at 1 and at 48 h for the three formulations were not significantly different. This may suggest that the liver is possibly the passive targeting tissue for the pegylated and regular liposomal doxorubicin. A similar situation was found in other investigations using mice (17, 18).

In vitro cytotoxicity demonstrated that the pegylated

liposomal doxorubicin and regular liposomal doxorubicin showed a higher efficacy than that of free doxorubicin, which may indicate that fatty-like lipids in liposomes containing doxorubicin may be beneficial for the cancer cell's uptake. Results from in vivo studies supported that the efficacies of pegylated liposomal and regular liposomal doxorubicin were significantly higher in comparison with that of free doxorubicin. Among these, the pegylated liposomal doxorubicin showed more favorable efficacies, as indicated by Harrington et al. (2) who found that pegylated liposomes accumulate more in tumors through leaky vessels present in tumors, thus enhancing the drug delivery efficiency. The concentrations after dosing the three formulations in solid tumor tissues were unavailable in present studies; thus further investigations for such a liposomal platform using topotecan are being undertaken in which the concentrations in solid tumor tissues are measured (data not shown). The treatment schemes seemed to affect the effectiveness, and the treatment time at the 7th day was better than that at 2nd day after inoculation of tumor cells. The possible reasons may be due to the fact that the doxorubicin concentrations decreased when the solid tumor just formed when using the 2nd day dosing schemes.

Pathological slices indicated that the free doxorubicin strongly eroded the gastric gland and mucosa, small intestine, and heart plasma myocytes. This may be the reason why vomiting, nausea, and heart toxicity appear after dosing in humans, as described in the Introduction. Pegylated and regular liposomal doxorubicin reduced the toxicity of the free drug to gastrointestinal and heart tissues; the possible reason may be due to the lowered concentrations in these tissues. Moreover, the toxic compound was encapsulated in the liposomes before being internalized, therefore alleviating the direct exposure and eroding of the tissues.

In conclusion, the pegylated liposomal doxorubicin markedly prolonged the blood circulation exposure of doxorubicin. The half-lives of the elimination phase in mice were 46.09 ± 14.44 h for pegylated liposomal doxorubicin, 26.04 ± 3.34 h for regular liposomal doxorubicin, and 23.72 ± 5.13 h for free doxorubicin. $AUC_{0-\infty}$ ($h \cdot \mu g/g$) of the pegylated and of the regular compounds were 6.8- and 2.6-fold higher than that of free doxorubicin. Concentrations of the pegylated or regular liposomal doxorubicin were significantly reduced in the stomach, small intestine, and heart tissues and increased in liver tissue in comparison with that of free doxorubicin. Cytotoxicity and antitumor activity in the xenograft tumor indicated that the pegylated liposomal doxorubicin showed a higher efficacy than regular liposomal doxorubicin or free

doxorubicin. After two weeks of tail intravenous injection of pegylated liposomal doxorubicin at a single dose of 10 mg/kg, no significant damage was observed in gastric mucosa, intestinal mucosa, and heart muscle. Both the pegylated and the regular doxorubicin reduced gastrointestinal toxicity and cardiotoxicity in comparison with free doxorubicin. As a control, pronounced damage was found in gastric mucosa including gland dilation and cell dropping along with topical inflammatory infiltration, in intestinal mucosa including denaturations of gland particles and Paneth's cells, and in heart muscle including fatty denaturation and eosinophilic-like plasma myocytes before necrosis. The results demonstrate that the pegylated liposomes improve the therapeutic efficacy of toxic compounds and reduce their toxicity, thus providing favorable evidence supporting the building of a pegylated liposomal platform.

References

- Cattel L, Ceruti M, Dosio F. From conventional to stealth liposomes: a new frontier in cancer chemotherapy. *Tumori*. 2003;89:237–249.
- Harrington KJ, Mohammadtaghi S, Uster PS, Glas D, Peters AM, Vile RG, et al. Effective targeting of solid tumors in patients with locally advanced cancers by radiolabeled pegylated liposomes. *Clin Cancer Res*. 2001;7:243–254.
- Ceh B, Winterhalter M, Frederik PM, Vallner JJ, Lasic D. Stealth liposomes: from theory to product. *Adv Drug Deliv Rev*. 1997;24:165–177.
- Gabizon A, Goren D, Barenholz Y. Investigations on the anti-tumor efficacy of liposome-associated doxorubicin in murine tumor models. *Isr J Med Sci*. 1988;24:512–517.
- Haran G, Cohen R, Bar LK, Barenholz Y. Transmembrane ammonium sulfate gradients in liposomes produce efficient and sTab. entrapment of amphipathic weak bases. *Biochim Biophys Acta*. 1993;115:201–215.
- Tardi P, Choice E, Masin D, Resdelmeier T, Bally M, Madden TD. Liposomal encapsulation of topotecan enhances anticancer efficacy in murine and human xenograft models. *Cancer Res*. 2000;60:3389–3393.
- Gabizon A, Shmieda1 H, Barenholz Y. Pharmacokinetics of pegylated liposomal doxorubicin: review of animal and human studies. *Clin Pharmacokinet*. 2003;42:419–436.
- Riggs ER Jr, Bachur R. Clinical pharmacokinetics of anthracycline antibiotics. In: Ames MM, Powis G, Kovach JS, editors. *Pharmacokinetics of anticancer agents in humans*. Amsterdam: Elsevier; 1983. p. 229–278.
- Chen ZP, McQuillan A, Mohr G, Panasci LC. Excision repair cross-complementing rodent repair deficiency gene 2 expression and chloroethylnitrosourea resistance in human glioma cell lines. *Neurosurgery*. 1998;42:1112–1119.
- Shinozawa S, Mimaki Y, Araki Y. Determination of the mass ratio of adriamycin and its metabolites in the serum and tissues of Ehrlich carcinoma-bearing mice by high-performance liquid chromatography. *J Chromatogr Biomed Appl*. 1980;196:463–469.
- Rose LM, Tillery KF, el Dareer SM, Hill DL. High-performance liquid chromatographic determination of doxorubicin and its metabolites in plasma and tissue. *J Chromatogr Biomed Appl*. 1988;425:419–423.
- Leca FR, Marchiset-Leca D, Antonetti M. A highly sensitive method for the determination of adriamycin and adriamycinol in human plasma using HPLC with fluorimetric detection. *Chromatographia*. 1989;28:325–328.
- Frezard F. Fluorometric determination in biological fluids of the release kinetics of liposome-entrapped doxorubicin. *J Liposome Res*. 1994;4:1063–1073.
- Hu OY, Chang SP, Jame JM, Chen KY. Pharmacokinetic and pharmacodynamic studies with 4'-epidoxorubicin in nasopharyngeal carcinoma patients. *Cancer Chemother Pharmacol*. 1989;24:332–337.
- Siegal T, Horowitz A, Gabizon A. Doxorubicin encapsulated in sterically stabilized liposomes for the treatment of a brain tumor model: biodistribution and therapeutic efficacy. *J Neurosurg*. 1995;83:1029–1037.
- Gabizon A, Barenholz Y, Bialer M. Prolongation of the circulation time of doxorubicin encapsulated in liposomes containing a polyethylene glycol-derivatized phospholipid: pharmacokinetic studies in rodents and dogs. *Pharm Res*. 1993;10:703–708.
- Gabizon A, Martin F. Polyethylene glycol-coated (pegylated) liposomal doxorubicin: rationale for use in solid tumors. *Drugs*. 1997;54 suppl 4:15–21.
- Vaage J, Donovan D, Uster P, Working P. Tumor uptake of doxorubicin in polyethylene glycol-coated liposomes and therapeutic effect against a xenografted human pancreatic carcinoma. *Br J Cancer*. 1997;75:482–486.