

Pharmacology & Toxicology

Pharmacology & Toxicology fields

Okayama University

Year 2008

Albumin-conjugated PEG liposome
enhances tumor distribution of liposomal
doxorubicin in rats

Jun-ichi Yokoe, *Pharmaceutical Research Division, NIPRO Corporation*

Shiho Sakuragi, *Pharmaceutical Research Division, NIPRO Corporation*

Kayoko Yamamoto, *Pharmaceutical Research Division, NIPRO Corporation*

Takuya Teragaki, *Department of Pharmaceutics, Faculty of Pharmaceutical Sciences, Okayama University*

Ken-ichi Ogawara, *Department of Pharmaceutics, Faculty of Pharmaceutical Sciences, Okayama University*

Kazutaka Higaki, *Department of Pharmaceutics, Faculty of Pharmaceutical Sciences, Okayama University*

Naohisa Katayama, *Pharmaceutical Research Division, NIPRO Corporation*

Toshiya Kai, *Pharmaceutical Research Division, NIPRO Corporation*

Makoto Sato, *Pharmaceutical Research Division, NIPRO Corporation*

Toshikiro Kimura, *Department of Pharmaceutics, Faculty of Pharmaceutical Sciences, Okayama University*

This paper is posted at eScholarship@OUDIR : Okayama University Digital Information Repository.

http://escholarship.lib.okayama-u.ac.jp/pharmacology_general/5

Research Paper

Albumin-conjugated PEG liposome enhances tumor distribution of liposomal doxorubicin in rats

Jun-ichi Yokoe^{1,2)}, Shiho Sakuragi¹⁾, Kayoko Yamamoto¹⁾, Takuya Teragaki²⁾, Ken-ichi Ogawara²⁾, Kazutaka Higaki²⁾, Naohisa Katayama¹⁾, Toshiya Kai¹⁾, Makoto Sato¹⁾ and Toshikuro Kimura²⁾

¹⁾ *Pharmaceutical Research Div., NIPRO Corporation, Shiga 525-0055 (Japan)*

²⁾ *Department of Pharmaceutics, Faculty of Pharmaceutical Sciences, Okayama University, Okayama 700-8530 (Japan)*

For mailing proof

Prof. Toshikuro Kimura

Department of Pharmaceutics, Faculty of Pharmaceutical Sciences,

Okayama University, 1-1-1 Tsushima-Naka, Okayama 700-8530, Japan

TEL (+81)-86-251-7948

FAX (+81)-86-251-7926

e-mail kimura@pharm.okayama-u.ac.jp

Abstract

To evaluate the effect of coupling of recombinant human serum albumin (rHSA) onto the surface of poly(ethylene glycol)-modified liposome (PEG liposome) on the in-vivo disposition characteristics of liposomal doxorubicin (DXR), the pharmacokinetics and tissue distribution of DXR were evaluated after intravenous administration of rHSA-modified PEG (rHSA/PEG) liposomal DXR into tumor-bearing rats. rHSA/PEG liposome prepared using a hetero-bifunctional cross-linker, N-succinimidyl 3-(2-pyridyldithio) propionate (SPDP), efficiently encapsulated DXR (over 95%). rHSA/PEG liposomal DXR showed longer blood-circulating property than PEG liposomal DXR and the hepatic and splenic clearances of rHSA/PEG liposomal DXR were significantly smaller than those of PEG liposomal DXR. It was also demonstrated that the disposition of DXR to the heart, one of the organs for DXR-related side-effects, was significantly smaller than free DXR. Furthermore, the tumor accumulation of rHSA/PEG liposomal DXR was significantly larger than that of PEG liposomal DXR. The “therapeutic index”, a criterion for therapeutic outcome, for rHSA/PEG liposomal DXR was significantly higher than PEG liposomal DXR. These results clearly indicate that rHSA-conjugation onto the surface of PEG liposome would be a useful approach to increase the effectiveness and safety of PEG liposomal DXR.

Keywords: recombinant human serum albumin (rHSA); PEG liposome; doxorubicin; tumor-bearing rats; passive targeting

1. Introduction

Liposomes containing either monosialoganglioside G_{M1} (Allen et al., 1989) or polyethylene glycol (PEG) derivatives (Blume and Cevc, 1990; Klivanov et al., 1990; Allen et al., 1991; Maruyama et al., 1992; Woodle and Lasic, 1992; Yuda et al., 1996) are not readily taken up by the macrophages in reticuloendothelial system (RES), and hence remain in the blood circulation for a relatively long period of time. Particularly, PEG-modified liposomes (PEG liposomes) have been utilized as a particulate carrier for anti-tumor therapy due to their long circulation time. Generally, as the capillary permeability of the endothelium in newly vascularized tumors is significantly greater than that of normal organs, long-circulating PEG liposomes are preferentially delivered and accumulated into the tumors (Enhanced permeability and retention (EPR) effect), which provides a great opportunity for passive targeting of liposomal anticancer agents into tumor tissues (Maeda et al., 2000; Luigi et al., 2003).

Doxorubicin hydrochloride (DXR) is the most commonly used anthracycline and is one of the most active agents in the treatment of breast cancer. However, it sometimes causes cardiotoxicity, which could lead to congestive heart failure and death (Dresdale et al., 1983; Speth et al., 1988). One of the approaches to avoid DXR-related toxicity is to encapsulate it into appropriate drug carriers, which provides a change in the in-vivo distribution of DXR, resulting in reduced DXR levels in the heart (Abraham et al., 2005). DXR encapsulated in PEG liposome, known as Doxil in the United States, has revealed an increased therapeutic efficacy and reduced cardiotoxicity compared to free DXR (Working and Dayan, 1996; Gabizon et al., 2003).

In the previous study, we reported that rat serum albumin (RSA)-conjugated PEG liposomes showed the longer circulation time than PEG liposomes after intravenous administration into rats (Furumoto et al., 2007), suggesting the potential of the albumin-conjugated PEG liposomes as a suitable carrier for various anticancer drugs. Albumin is one of the endogenous, non-toxic, non-immunogenic and relatively hydrophilic proteins in the body. Its introduction on the surface

of liposomes reduced the association of serum proteins including some given serum opsonins onto the surface, resulting in the more prolonged circulation time of PEG liposome (Furumoto et al., 2007). However, since the conjugation of rat serum albumin onto PEG liposome with carbodiimide has to be conducted under weakly acidic condition, the pH remote loading method (Mayer et al., 1986; Madden et al., 1990), which can encapsulate DXR into the liposome very efficiently, was not available. In the present study, we changed the method for albumin conjugation in order to encapsulate DXR into liposomes by utilizing the pH remote loading method. N-succinimidyl 3-(2-pyridyldithio) propionate (SPDP) (Carlsson et al., 1978) was selected as a hetero-bifunctional cross-linking agent to couple recombinant human serum albumin (rHSA) onto PEG liposome. This method allowed us to employ the pH remote loading of DXR into the liposome. Then, the effect of rHSA-conjugation to PEG liposome was evaluated in terms of pharmacokinetics and biodistribution of liposomal DXR in Yoshida sarcoma (LY-80)-bearing rats.

2. Materials and methods

2.1. Materials

Egg phosphatidylcholine (egg PC) was purchased from ASAHI KASEI Chemicals Industry Inc. (Tokyo, Japan). Cholesterol (Chol), doxorubicin hydrochloride (DXR) and daunorubicin hydrochloride were obtained from Wako Pure Chemical Industry Inc. (Osaka, Japan). Dioleoyl phosphatidylethanolamine (DOPE) and distearoylphosphatidylethanolamine-N-[methoxy poly (ethylene glycol)-2000] (PEG-DSPE) were purchased from NOF Inc. (Tokyo). SPDP was purchased from PIERCE Inc. (Rockford, IL, USA). rHSA was gifted from Bipla Inc. (Chitose, Japan).

2.2. Synthesis of PDP-DOPE, as a linker of rHSA-conjugation with liposomes

We synthesized a DOPE derivatized with terminal pyridyldithiopropionate (PDP) groups as

previously reported (Barbet et al., 1981; Ishimori et al., 1984). Mixture of DOPE in chloroform and SPDP in methanol (SPDP:DOPE = 67.2:78 molar ratio) were stirred for 2 hr under nitrogen gas at room temperature after a small aliquot of triethylamine was added to the mixture. To remove unreacted SPDP, the organic phase was reverse-extracted with phosphate buffered saline (PBS, pH 7.4) three times. After the organic solvent was evaporated, the residue was re-dissolved in chloroform to give a final concentration of 10 μmol PDP-DOPE/mL. Thin layer chromatography (solvent; chloroform:methanol:water = 65:25:4 molar ratio) on silica gel indicated a single spot under UV illumination.

2.3. Preparation of liposomes

Liposomes were prepared according to the following procedures. Lipid mixture (Egg PC:Chol:PEG-DSPE:PDP-DOPE = 61:30:5:4 molar ratio) was dried by rotary evaporator at 40°C. Two-hundred and fifty mM ammonium sulfate solution (pH 5.5) was added to the thin-film of lipids and the mixture was hydrated at 60°C. The resultant suspension was extruded at least 10 times through polycarbonate membranes of 100 nm pore size (Whatman plc., Brentford, UK). External solution was replaced with PBS (pH 8.0) by gel filtration with a Sephadex G-25 column (PD-10, GE Healthcare Ltd., Buckinghamshire, UK).

DXR was encapsulated into liposome using the pH remote loading method (Bolotin et al., 1994). DXR was dissolved at 7 mg/mL in PBS (pH 8.0), and then, immediately mixed with liposomal suspension and incubated at 60°C for 1 hr. Non-encapsulated DXR was removed with a PD-10 column equilibrated with PBS (pH 8.0). The extent of DXR encapsulation was determined by measuring liposomal DXR amount by HPLC method as described below.

2.4. Coupling of PDP-rHSA to liposomes

SPDP in methanol was added to rHSA dissolved in PBS (SPDP:rHSA = 20:1 molar ratio)

under nitrogen gas and the mixture was incubated at room temperature for 30 min. Excess of SPDP was then removed by Sepharose CL-6B column (Bio-Rad, Emeryville, CA) equilibrated with acetate buffer (pH 4.5). PDP-rHSA was incubated with dithiothreitol (DTT) (PDP-rHSA:DTT = 1:250 molar ratio) for 20 min at room temperature and the reaction mixture was applied to a Sepharose CL-6B column equilibrated with PBS (pH 8.0) to remove unreacted DTT. The activated rHSA in the elution was dropped to liposomal suspensions and the mixture was left at least for 18 hr at room temperature under nitrogen gas with gentle stirring. Unreacted rHSA was removed by Sepharose CL-6B column equilibrated with PBS (pH 8.0). As a control, “PEG liposome” was also prepared by coupling cysteine instead of rHSA.

2.5. Physicochemical characterization of rHSA-conjugated PEG liposomes (rHSA/PEG liposomes) encapsulating DXR

The diameter of the liposomes was determined by light-scattering spectroscopy using a NICOMP zls-370 (Particle Sizing System, Santa Barbara, CA). Lipid and rHSA amounts were estimated by using Phospholipid B test Wako and albumin test Wako (Wako Pure Chemical Industries, Osaka), respectively. Each liposome sample was subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE) as previously reported (Furumoto et al., 2007).

In-vitro stability of liposome encapsulating DXR was tested as follows: DXR-encapsulated liposomal suspensions were incubated with the same volume of rat plasma at 37°C for 2 hr. Then, the released DXR was separated by the Sepharose CL-6B column and was determined spectrofluorometrically (Ex = 500 nm, Em = 550 nm).

2.6. Determination of the amount of serum proteins associated with liposomes

Liposomes were incubated in rat serum (liposomal suspension:serum = 1:1, v/v) for 20 min at 37 °C, and subsequently bulk serum proteins were removed by Sepharose CL-4B column (Bio-Rad,

Emeryville, CA). The amount of serum proteins associated with liposomes was calculated by subtracting the amount of rHSA coupled with liposomes from the amount of total protein quantified by Lowry's method (Lowry et al., 1951).

2.7. Animals

Male Donryu rats weighing 200-280 g (Charles River Laboratories Inc., Yokohama, Japan) were used throughout the present study. Rats were maintained at 23°C and 55% of humidity with free access to standard rat food and water. Our investigations were performed after approval by our local ethical committee at Nipro Corporation and Okayama University, and in accordance with Principles of Laboratory Animal Care (NIH publication #85-23).

2.8. In-vivo disposition experiments

LY-80, rat ascites sarcoma cell line was kindly provided from Cell Resource Center for Biomedical Research, Institute of Development, Aging and Cancer, Tohoku University (Sendai, Japan). Rats were subcutaneously inoculated into the thigh with 1.0×10^6 LY-80 cells in a volume of 0.1 mL. Liposomal DXR or free DXR was intravenously injected at 2.0 mg/kg as DXR around 7 days after the tumor inoculation, when the tumor grew up to about 600 mm³ in volume. Then, blood samples were periodically taken from the cannulated jugular vein. Blood samples (0.2 mL) were centrifuged immediately at 4,000 g for 5 min and the obtained plasma samples were kept at -20°C until analysis. Tissue distribution studies were conducted as follows. At 3 hr after intravenous injection, organs (liver, spleen, heart and tumor) were excised, rinsed with PBS, and weighed. All tissues were stored at -20°C until analysis.

2.9. Analytical method

DXR was extracted from plasma and tissue samples as previously reported (Bally et al., 1990;

Embee et al., 1993). In brief, 0.1 mL of plasma was added to 0.9 mL of saturated ammonium sulfate (pH 4.0 buffered saline) with daunorubicin, an internal standard. Subsequently, the sample was extracted with 2 mL of chloroform/isopropanol (1:1, v/v). Following vigorous mixing, 1,600 g-centrifugation and the evaporation of organic phase, the residue was re-dissolved in HPLC mobile phase. In the case of tissue samples, after each tissue was homogenized with PBS (pH 7.4) (3.0 g/mL), 1 mL of the homogenate was subjected to the same procedure used for plasma samples.

The HPLC system was composed of LC-10AS pump, SIL-10A autosampler, RF-10A fluorescence detector (Shimadzu, Kyoto, Japan) set at Ex = 500 nm and Em = 550 nm. An ODS column (5C₁₈, 150 × 4.6 mm I.D, Nacalai Tesque, Inc., Kyoto) was used at room temperature. The mobile phase was 1/15 M KH₂PO₄ : CH₃CN = 75 : 25 (v/v, pH 4.16, adjusted with H₃PO₄), which was delivered at 1.0 mL/min. The coefficient of variation (CV) for standard curves ranged from 2.3 to 5.6 and the squared correlation coefficient was over 0.99.

2.10. Pharmacokinetic analysis

Plasma concentrations of DXR (C_p) versus time curves were analyzed by Eq. (1) using the non-linear least-square regression program MULTI (Yamaoka et al., 1981)

$$C_p = A \cdot e^{-\alpha t} + B \cdot e^{-\beta t} \quad (1)$$

The area under the plasma concentration-time curve (AUC) was calculated by Eq. (2).

$$AUC = \int_0^t C_p dt \quad (t = \infty) \quad (2)$$

Tissue clearance (CL_{tissue}) was calculated by Eq. (3)

$$CL_{tissue} = \frac{X_0^t}{AUC_0^t} \quad (t = 3 \text{ hr}) \quad (3)$$

where AUC_0^t means AUC value from 0 to time t, and X_0^t represents the amount of liposomes in a tissue at time t.

2.11. Statistical analysis

Results are expressed as the mean \pm S.D. Analysis of variance (ANOVA) was used to test the statistical significance of differences among groups. Statistical significance in the difference of the means was evaluated by using Student's *t*-test or Dunnett's test for the single or multiple comparisons of experimental groups, respectively.

3. Results

Several physicochemical characteristics were evaluated for DXR-encapsulated rHSA/PEG liposome and PEG liposome. The average diameters of DXR-encapsulated rHSA/PEG and PEG liposomes were 94.4 ± 6.4 nm and 95.5 ± 8.1 nm, respectively. DXR encapsulation efficiencies were 97.4 ± 1.4 % and 95.8 ± 2.1 % for rHSA/PEG liposome and PEG liposome, respectively. The amount of rHSA conjugated onto the surface of PEG liposome was 5.3 ± 1.6 $\mu\text{g}/\mu\text{mol}$ total lipid. SDS-PAGE analysis under non-reducing condition revealed that rHSA coupled onto the surface of liposome was exclusively in a monomeric form (data not shown). The release of DXR from the two PEG liposomal preparations was evaluated in an in vitro study for 2 hr at 37°C. The released fraction of DXR was 2.3 ± 2.6 % or 9.3 ± 5.4 for rHSA/PEG or PEG liposome, respectively.

The in-vivo disposition of DXR was evaluated after intravenous injection of free DXR, PEG liposomal DXR or rHSA/PEG liposomal DXR into tumor-bearing rats at a dose of 2.0 mg/kg as DXR. The plasma concentration-time curves of DXR after intravenous administration of each preparation were shown in Fig. 1 and pharmacokinetic parameters of DXR were summarized in Table 1. Figure 1 clearly shows that the injection of rHSA/PEG liposomal DXR exhibited much higher plasma concentrations of DXR compared with free DXR injection, and moreover significantly higher than the injection of PEG liposomal DXR. AUC of DXR for rHSA/PEG

liposome ($89.7 \pm 12.9 \mu\text{g}\cdot\text{hr}/\text{mL}$) was significantly larger than that for PEG liposome ($33.8 \pm 2.8 \mu\text{g}\cdot\text{hr}/\text{mL}$), but both values of AUC were extensively larger than that for free DXR ($4.52 \pm 0.69 \mu\text{g}\cdot\text{hr}/\text{mL}$). Total body clearance (CL_{total}), distribution volume (V_d) and elimination rate constant (k_{el}) of DXR for both PEG liposomal preparations were significantly smaller than those for free DXR. Furthermore, rHSA/PEG liposome provided significantly smaller CL_{total} and k_{el} of DXR than PEG liposome. These results clearly indicate that rHSA-conjugation prolongs the residence time of PEG liposomal DXR in blood circulation.

In the in-vivo disposition study, the distribution of DXR after intravenous administration of each preparation was investigated for liver, spleen and heart (Fig. 2), because liver and spleen are main organs for liposome disposition and cardiotoxicity is a critical side-effect of DXR. At 3 hr after injection, distribution of DXR into RES was larger for both PEG liposomal preparation than that for free DXR, but the hepatic and splenic clearances were remarkably smaller for both liposome preparations than that for free DXR, suggesting that rHSA/PEG and PEG liposomal preparations would suppress and delay the uptake of DXR into RES. Furthermore, it was revealed that the hepatic and splenic clearances of DXR for rHSA/PEG liposome were significantly smaller than those for PEG liposome, suggesting that the affinity of rHSA/PEG liposome to these organs would be less than PEG liposome. In addition, PEG liposomal preparations significantly suppressed the distribution of DXR into heart compared with free DXR, although there was no significant difference between rHSA/PEG liposome and PEG liposome (Figs. 2 and 3).

To obtain some clue to explain the reason for the lower hepatic and splenic clearances of rHSA/PEG liposomal DXR than PEG liposomal DXR, we measured the amount of serum proteins associated onto the surface of rHSA/PEG or PEG liposomes (Fig. 3). The result clearly demonstrated that the amount of associated serum proteins was significantly reduced by the rHSA-conjugation.

Next, we evaluated the disposition of DXR into tumor tissue at 3 hr after intravenous

administration (Fig. 4). Significantly increased tumor distribution of DXR was observed for both rHSA/PEG and PEG liposomal preparations compared with free DXR. Furthermore, it should be noted that the amount of DXR in tumor was significantly larger for rHSA/PEG liposome than PEG liposome, demonstrating the usefulness of rHSA-conjugation onto PEG liposome for the better DXR delivery into tumor tissues.

The therapeutic outcome of DXR would be evaluated by the balance between its anti-tumor effect and side-effect. Therefore, as a criterion for therapeutic outcome, the therapeutic index, tumor to heart ratio of DXR amount, was calculated for both PEG liposomal preparations and free DXR (Fig. 4). The therapeutic index of rHSA/PEG liposomal DXR was the largest among the three preparations, although PEG liposomal DXR was also significantly better than free DXR. This result clearly demonstrates that rHSA/PEG liposomal DXR would provide better EPR effect for tumor tissues than PEG liposomal DXR.

4. Discussion

Long-circulating particles are promising carriers for passive targeting of drugs into tumors or inflamed tissues, where the integrity of the endothelial barrier is perturbed, via EPR effect (Gabizon and Papahadjopoulos, 1992; Jang et al., 2003). In this study, we tried to evaluate the pharmacokinetics and biodistribution of DXR encapsulated into rHSA-conjugated PEG liposome in tumor-bearing rats.

Several factors such as particle size, charge and lipid composition of liposome have been reported to influence the in-vivo fate of liposomes after intravenous administration in rats (Gabizon et al., 1993; Harashima et al., 2002). Among them, the size of liposome is one of the most important factors to influence the EPR effect-driven tumor disposition and the liposomes with the diameter of less than 150 nm are reported to be suitable for the efficient delivery (Harashima and Kiwada, 1996; Drummond et al., 1999; Takeuchi et al., 2001). Therefore, we decided to prepare

liposomes with a diameter of 100 nm.

In our previous study, we clearly demonstrated that RSA-conjugated PEG liposome (RSA/PEG liposome), prepared by using carbodiimide, prolonged the blood circulation time of PEG liposome after intravenous administration in rats (Furumoto et al., 2007). Carbodiimide has been widely used as carboxyl- and amine-reactive crosslinker to prepare immunoliposomes (Endoh et al., 1981), polymer-protein conjugates (Dilgimen et al., 2001) and immunomicrospheres (MacAdam et al., 2000). However, the pH remote loading method is not available for DXR encapsulation into the albumin-conjugated PEG liposome, since carbodiimide can activate carbonyl group only at a low pH region, especially between 3.5 and 4.5 during the first step of reaction (Nakajima and Ikeda, 1995). Therefore, in the present study, we chose SPDP as the heterobifunctional-crosslinker (Carlsson et al., 1978) to prepare rHSA/PEG liposomes, because the coupling reaction can be conducted under weakly alkaline pH, which makes it possible to perform the pH remote loading method for encapsulating DXR into rHSA/PEG liposomes. SPDP has been widely used for the preparation of disulphide-linked protein-protein conjugates (Moll and Thompson, 1994; Takeoka et al., 2001) and immunoliposome (Berbet et al., 1981; Ishimori et al., 1984; Schwendener et al., 1990). As a result, the encapsulation efficiency of DXR into rHSA/PEG liposomes or PEG liposomes was very high and more than 95% of DXR added was successfully encapsulated.

In-vivo disposition studies in tumor-bearing rats revealed that the DXR encapsulation into rHSA/PEG liposomes or PEG liposomes dramatically changed the in-vivo disposition characteristics of DXR itself (Fig. 1 and Table 1). Furthermore, rHSA-conjugation onto the surface of liposomes significantly prolonged the blood circulation of DXR compared with PEG liposomal DXR. Taken that the in-vitro release study demonstrated that DXR was stably and similarly encapsulated within both liposomal preparations in the presence of plasma and that free DXR was immediately cleared from the blood circulation after injection (Fig. 1), the in-vivo disposition characteristics of rHSA/PEG liposomal DXR or PEG liposomal DXR is considered to

mainly represent the pharmacokinetics of each PEG liposomes themselves. In addition, rHSA/PEG liposomes significantly reduced the hepatic and splenic clearances of DXR compared with PEG liposomes, although both PEG liposomal preparations remarkably decreased both tissue clearances compared with free DXR (Fig. 2B). Although the disposition amount of DXR in the liver and spleen was larger for both rHSA/PEG and PEG liposomal DXR than that for free DXR, it would be because the distribution of DXR into and the subsequent elimination of DXR from the liver is delayed and prolonged for liposomal preparations, compared with free DXR. The distribution of free DXR to and its subsequent elimination from the liver due to the metabolism, biliary excretion or efflux back to the blood stream are very fast. (Colombo et al., 1994, Working et al., 1996). In the case of heart, the amount of DXR at 3 hr would reflect the accumulation of DXR in heart (Figs. 2 and 4), considering the very rapid elimination of free DXR from plasma (Fig. 1). Clearance values also confirmed that both PEG liposomal preparations significantly attenuated DXR distribution to heart (Fig. 2B). As Speth et al. (1998) reported that one of the acute or delayed toxicities derived from DXR was cardiac arrhythmias or cardiomyopathy, respectively, the decrease in DXR distribution to heart would be one of the advantages rHSA/PEG liposome can provide.

As described above, in-vivo disposition studies clearly indicated the longer circulation of rHSA/PEG liposomal DXR and lower values of tissue clearances for liver and spleen than PEG liposomal DXR. The serum proteins associated onto the surface of liposomes systematically administered have been suggested to be one of the most important factors to determine their in-vivo fate (Drummond et al., 1999; Luigi et al., 2003). Chonn et al. (1992) reported that the amount of serum proteins associated on the liposomes used was inversely related to their circulation half-lives. Our present findings also revealed that less amount of serum proteins was associated on rHSA/PEG liposomes than PEG liposomes (Fig. 3). Since the recognition of surface-associated serum opsonins by their corresponding receptors is mainly a trigger for the receptor-mediated hepatic

uptake of liposomes, it is considered that the rHSA-conjugation on PEG liposome suppressed the association of serum proteins including some serum opsonins. Western blotting will be useful to address the possible less-association of typical serum opsonins on rHSA/PEG liposome and will be the subject of our further study.

The movement of liposomes into the tumor interstitium is principally via extravasation through the discontinuous endothelium of the tumor microvasculature (Drummond et al., 1999). Since the maintenance of high blood level or large AUC of particulate drug carriers is one of the driving forces for the efficient extravasation into tumor tissues (Drummond et al., 1999), it can be considered that rHSA/PEG liposomal preparation successfully improved the tumor disposition of DXR over PEG liposomal preparation via EPR effect (Fig. 4). For DXR to exert the anti-tumor effect, DXR must be released and taken up by the surrounding tumor cells. Although DXR is stably incorporated in rHSA/PEG liposomes in plasma, DXR release from the liposome preparations would be enhanced after liposomes are extravasated into tumor tissues, considering that malignant effusions significantly elevated the release of DXR from liposome preparation (Gabizon A.A., 1995). Elucidation of local release profile of DXR in tumor tissues will be the subject for further study.

From the viewpoint of clinical therapeutics, the balance between pharmacological and adverse effects is very important. Therefore, we calculated the therapeutic index defined as the ratio of the amount of DXR delivered to tumor, the site of action, to the DXR amount at potential sites of toxicity, heart (Fig. 4). Therapeutic index was the highest for rHSA/PEG liposomal DXR, suggesting that rHSA-conjugation on PEG liposome would increase the anti-tumor effect as well as the safety of liposomal DXR.

In conclusion, rHSA modification on the surface of PEG liposome significantly prolonged the blood circulation time of PEG liposomal DXR, leading to higher DXR amount in the tumor, but lower level of DXR in heart after intravenous administration. These findings are very useful to

optimize albumin-conjugated PEG liposome for the passive targeting of encapsulated drug and for the better therapeutic outcome.

References

- Abraham, S.A., Waterhouse, D.N., Mayer, L.D., Cullis, P.R., Madden, T.D., Bally, M.B., 2005. The liposomal formulation of doxorubicin. *Methods. Enzymol.*, 391, 71-97.
- Allen, T.M., Hansen, C., Rutledge, J., 1989. Liposomes with prolonged circulation times: factors affecting uptake by reticuloendothelial and other tissues. *Biochim. Biophys. Acta*, 981, 27-35.
- Allen, T.M., Hansen, C., Martin, F., Redemann, C., Yau-Young, A., 1991. Liposomes containing synthetic lipid derivatives of poly(ethylene glycol) show prolonged circulation half-lives in vivo. *Biochim. Biophys. Acta*, 1066, 29-36.
- Bally, M.B., Nayar, R., Masin, D., Hope, M.J., Cullis, P.R., Mayer, L.D., 1990. Liposomes with entrapped doxorubicin exhibit extended blood residence times. *Biochim. Biophys. Acta*, 1023, 133-139.
- Barbet, J., Machy, P., Leserman, L.D., 1981. Monoclonal antibody covalently coupled to liposomes: specific targeting to cells. *J. Supramol. Struct. Cell Biochem.*, 16, 243-258.
- Blume, G., Cevc, G., 1990. Liposomes for the sustained drug release in vivo. *Biochim. Biophys. Acta*, 1029, 91-97.
- Bolotin, E.M., Cohen, R., Bar, L.K., Emanuel, S.N., Lasic, D.D., Barenholz, Y., 1994. Ammonium sulfate gradients for efficient and stable remote loading of amphipathic weak bases into liposomes and ligandliposomes. *J. Liposome Res.*, 4, 455-479.

Carlsson, J., Drevin, H., Axen, R., 1978. Protein thiolation and reversible protein-protein conjugation. N-Succinimidyl 3-(2-pyridyldithio) propionate, a new heterobifunctional reagent. *Biochem. J.*, 173, 723-737.

Chonn, A., Semple, S.C., Cullis, P.R., 1992. Association of blood proteins with large unilamellar liposomes in vivo: Relation to circulation lifetimes. *J. Biol. Chem.*, 267, 18759-18765.

Colombo, T., Zucchetti, M., D'Incalci, M., 1994. Cyclosporin A markedly changes the distribution of doxorubicin in mice and rats. *J. Pharmacol. Exp. Ther.*, 69, 22-27.

Dilgimen, A.S., Mustafaeva, Z., Demchenko, M., Kaneko, T., Osada, Y., Mustafaev, M., 2001. Water-soluble covalent conjugates of bovine serum albumin with anionic poly(N-isopropyl-acrylamide) and their immunogenicity. *Biomaterials*, 22, 2383-2392.

Dresdale, A., Bonow, R.O., Wesley, R., Palmeri, S.T., Barr, L., Mathison, D., D'Angelo, T., Rosenberg, S.A., 1983. Prospective evaluation of doxorubicin-induced cardiomyopathy resulting from postsurgical adjuvant treatment of patients with soft tissue sarcomas. *Cancer*, 52, 51-60.

Drummond, D.C., Meyer, O., Hong, K., Kirpotin, D.B., Papahadjopoulos, D., 1999. Optimizing liposomes for delivery of chemotherapeutic agents to solid tumors. *Pharmacol. Rev.*, 51, 691-743.

Endoh, H., Suzuki, Y., Hashimoto, Y., 1981. Antibody coating of liposomes with 1-ethyl-3-(3-dimethyl-aminopropyl)carbodiimide and the effect on target specificity. *J. Immunol. Methods*, 44, 79-85.

Furumoto, K., Yokoe, J., Ogawara, K., Amano, S., Takaguchi, M., Higaki, K., Kai, T., Kimura, T., 2007. Effect of coupling of albumin onto surface of PEG liposome on its in vivo disposition. *Int. J. Pharm.*, 329, 110-116.

Gabizon, A., Papahadjopoulos, D., 1992. The role of surface charge and hydrophilic groups on liposome clearance in vivo. *Biochim. Biophys. Acta*, 1103, 94-100.

Gabizon, A.A., Barenholz, Y., Bialer, M., 1993. Prolongation of the circulation time of doxorubicin encapsulated in liposomes containing a polyethylene glycol-derivatized phospholipid: pharmacokinetic studies in rodents and dogs. *Pharm. Res.*, 10, 703-708.

Gabizon A.A., 1995. Liposome circulation time and tumor targeting: Implications for cancer chemotherapy. *Adv. Drug Delivery Review*, 16, 285-294.

Gabizon, A., Shmeeda, H., Barenholz, Y., 2003. Pharmacokinetics of pegylated liposomal doxorubicin: review of animal and human studies. *Clin. Pharmacokinet.*, 42, 419-436.

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

Harashima, H., Ishida, T., Kamiya, H., Kiwada, H., 2002. Pharmacokinetic of targeting with liposomes. *Crit. Rev. Ther. Drug Carrier Syst.*, 19, 235-275.

Ishimori, Y., Yasuda, T., Tsumita, T., Notsuki, M., Koyama, M., Tadakuma, T., 1984. Liposome immune lysis assay (LILA): a simple method to measure anti-protein antibody using protein

antigen-bearing liposomes. *J. Immunol. Methods*, 75, 351-360.

Jang, S.H., Wientjes, M.G., Lu, D., Au, J.L.S., 2003. Drug delivery and transport to solid tumors. *Pharm. Res.*, 20, 1337-1350.

Klibanov, A.L., Maruyama, K., Torchilin, V.P., Huang, L., 1990. Amphipathic polyethyleneglycols effectively prolong the circulation time of liposomes. *FEBS Lett.*, 268, 235-237.

Lowry O.H., Rosebrough N.J., Farr A.L., Randall R.J., 1951, Protein measurement with the Folin phenol reagent, *J. Biol. Chem.* 193, 265-75.

Luigi, C., Maurizio, C., Franco, D., 2003. From conventional to stealth liposomes. A new frontier in cancer chemotherapy. *Tumori*, 89, 237-249.

MacAdam, A.B., Shafi, Z.B., Marriott, C., Martin, G.P., James, S.L., 2000. Anti-mucus polyclonal antibody production, purification and linkage to the surface of albumin microspheres. *Int. J. Pharm.*, 195, 147-158.

Madden, T.D., Harrigan, P.R., Tai, L.C., Bally, M.B., Mayer, L.D., Redelmeier, T.E., Loughrey, H.C., Tilcock, C.P., Reinish, L.W., Cullis, P.R., 1990. The accumulation of drugs within large unilamellar vesicles exhibiting a proton gradient: a survey. *Chem. Phys. Lipids.*, 53, 37-46.

Maeda, H., Wu, J., Sawa, T., Matsumura, Y., Hori, K., 2000. Tumor vascular permeability and the EPR effect in macromolecular therapeutics. *J. Control. Release*, 65, 271-284.

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

Mayer, L.D., Bally, M.B., Cullis, P.R., 1986. Uptake of adriamycin into large unilamellar vesicles in response to a pH gradient. *Biochim. Biophys. Acta*, 857, 123-126.

Moll, T.S., Thompson, T.E., 1994. Semisynthetic proteins: model systems for the study of the insertion of hydrophobic peptides into preformed lipid bilayers. *Biochemistry*, 33, 15469-15482.

Nakajima, N., Ikada, Y., 1995. Mechanism of amide formation by carbodiimide for bioconjugation in aqueous media. *Bioconjug. Chem.*, 6, 123-130.

Schwendener, R.A., Trub, T., Schott, H., Langhals, H., Barth, R.F., Groscurth, P., Hengartner, H., 1990. Comparative studies of the preparation of immunoliposomes with the use of two bifunctional coupling agents and investigation of in vitro immunoliposome-target cell binding by cytofluorometry and electron microscopy. *Biochim. Biophys. Acta*, 1026, 69-79.

Speth, P.A., van Hoesel, Q.G., Haanen, C., 1988. Clinical pharmacokinetics of doxorubicin. *Clin. Pharmacokinet.*, 15, 15-31.

Takeoka, S., Teramura, Y., Okamura, Y., Handa, M., Ikeda, Y., Tsuchida, E., 2001. Fibrinogen-conjugated albumin polymers and their interaction with platelets under flow conditions. *Biomacromolecules*, 2, 1192-1197.

Takeuchi, H., Kojima, H., Yamamoto, H., Kawashima, Y., 2001. Passive targeting of doxorubicin with polymer coated liposomes in tumor bearing rats. *Biol. Pharm. Bull.*, 24, 795-799.

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

Working, P.K., Dayan, A.D., 1996. Pharmacological-toxicological expert report. CAELYX. (Stealth liposomal doxorubicin HCl). *Hum. Exp. Toxicol.*, 15, 751-785.

Yamaoka, K., Tanigawara, Y., Nakagawa, T., Uno, T., 1981. A pharmacokinetic analysis program (multi) for microcomputer. *J. Pharmacobiodyn.*, 4, 879-885.

Yuda, T., Maruyama, K., Iwatsuru, M., 1996. Prolongation of liposome circulation time by various derivatives of polyethyleneglycols. *Biol. Pharm. Bull.*, 19, 1347-1351.

Legends for Figures

Fig. 1 Plasma concentration – time profiles of DXR after intravenous administration of free DXR, PEG liposomal DXR or rHSA/PEG liposomal DXR to tumor-bearing rats. Each preparation was dosed at 2.0 mg/kg as DXR. Keys; ●, rHSA/PEG liposomal DXR; ■, PEG liposomal DXR; ▲, free DXR. Results are expressed as the mean \pm S.D. of three experiments. * $p < 0.05$; ** $p < 0.01$, compared with PEG liposomal DXR. † $p < 0.05$; †† $p < 0.01$, compared with free DXR.

Fig. 2 Tissue distribution of DXR after intravenous administration of free DXR, PEG liposomal DXR or rHSA/PEG liposomal DXR to tumor-bearing rats. (A) Distributed amount of DXR at 3 hr after intravenous administration. (B) Tissue clearance calculated according to equation (3). Each preparation was dosed at 2.0 mg/kg as DXR. Keys; □, free DXR; ▨, PEG liposomal DXR; ■, rHSA/PEG liposomal DXR. Results are expressed as the mean \pm S.D. of three experiments. * $p < 0.05$, compared with PEG liposomal DXR in each tissue. † $p < 0.05$; †† $p < 0.01$, compared with free DXR in each tissue.

Fig. 3 Amount of serum proteins associated on surface of PEG liposome and rHSA/PEG liposome prepared by SPDP method. Results are expressed as the mean \pm S.D. of three experiments. * $p < 0.05$; compared with PEG liposome. The amount of serum proteins associated with rHSA/PEG liposome was calculated by subtracting the amount of rHSA coupled with liposomes from the total protein amount measured.

Fig. 4 Amounts of DXR in tumor and heart at 3 hr (left) and therapeutic index (right) of

free DXR, PEG liposomal DXR or rHSA/PEG liposomal DXR after intravenous administration to tumor-bearing rats. Each preparation was dosed at 2.0 mg/kg as DXR. Therapeutic index was calculated as the tumor to heart ratio of DXR amount. Keys; \boxtimes , DXR amount in the tumor; \square , DXR amount in the heart; \blacksquare , therapeutic index of each formulation. Results are expressed as the mean \pm S.D. of three experiments. * $p < 0.05$, compared with PEG liposomal DXR. $\dagger p < 0.05$, compared with free DXR.

Table 1 Pharmacokinetic parameters of DXR after intravenous administration of free DXR, PEG liposomal DXR or rHSA/PEG liposomal DXR to tumor-bearing rats

	AUC ($\mu\text{g}\cdot\text{hr}/\text{mL}$)	CL_{total} (mL/hr)	V_d (mL)	k_{el} (hr^{-1})
Free DXR	4.52 ± 0.69	131.0 ± 16.1	27.8 ± 5.5	4.86 ± 1.5
PEG liposomal DXR	$33.8 \pm 2.8^{\dagger\dagger}$	$17.9 \pm 1.0^{\dagger\dagger}$	$19.5 \pm 4.4^{\dagger}$	$0.95 \pm 0.17^{\dagger\dagger}$
rHSA/PEG liposomal DXR	$89.7 \pm 12.9^{*,\dagger\dagger}$	$7.0 \pm 1.2^{*,\dagger\dagger}$	$14.9 \pm 0.9^{\dagger}$	$0.47 \pm 0.08^{*,\dagger}$

Each preparation was dosed at 2.0 mg/kg as DXR. AUC, area under the plasma-concentration time curve; CL_{total} , total clearance; V_d , apparent distribution volume; and k_{el} , elimination rate constant, were calculated based on two-compartment model. Results are expressed as the mean \pm S.D. of three experiments. * $p < 0.05$, compared with PEG liposomal DXR. † $p < 0.05$; †† $p < 0.01$, compared with free DXR.