Anticancer Drug-Phospholipid Conjugate for Enhancement of Intracellular Drug Delivery

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Summary: Tumor specific delivery of anti-cancer drugs is one of the major challenges faced by drug development processes. In this study, we prepared a doxorubicin (DOX)-conjugated liposome (DCL) by incorporating the newly synthesized DSPE-PEG2000-DOX (DPD) into liposomes as a lipid component and tested its anti-tumor activity *in vivo*. DPD was synthesized by coupling DOX to DSPE-PEG2000-COOH via amide linkage and the chemical structure of resulting DPD was confirmed by ^{1}H-NMR analysis. DCL having liposome size of 130 nm was prepared through thin film cast-hydration method. DCL was found to have significantly higher cellular uptake than conventional liposomes as confirmed by flow cytometry analysis. Anti-tumor activity of DCL against murine B16F10 melanoma tumor-bearing mice revealed that DCL inhibits tumor growth more efficiently than the conventional liposomes, presumably attributed to DOX mediated endocytosis process.

Keywords: cancer; chemotheraphy; doxorubicin; liposomes; targeting

Introduction

Liposomes have been extensively investigated as drug carriers for a variety of drugs including anticancer drugs.[1,2] Liposomal drugs are usually administrated through intravenous injection for systemic applications. In order to increase therapeutic efficacy of liposomal formulation of anti-cancer drug, polyethyleneglycol (PEG)-coated liposomes for prolongation of circulation half-life time or cancer cell-targetable liposomes having a cancer cell specific motifs have been studied.[3] Anthracycline drugs including doxorubicin (DOX) are known to readily intercalate into DNA strands[4] and many studies have shown that DOX preferentially accumulates into the nuclear compartment of cells.[5,6]

In this study, an anthracycline drug, DOX, was conjugated to the end-group of carboxylated phospholipids. Doxorubicin-conjugated liposomes (DCL) (see Figure 1(A)) were prepared by incorporating DSPE-PEG2000-DOX (DPD) as one of the lipid component into liposomes. Cellular uptake of DCL (Figure 1(B)) was evaluated through flow cytometry analysis and was compared to that of conventional liposomes. The antitumor activity *in vivo* was evaluated in a subcutaneous B16F10 melanoma xenograft model.

Experimental Part

Materials

L-α-Phosphatidylcholine(soy-hydrogenated) (HSPC), cholesterol (CHOL) and 1, 2-

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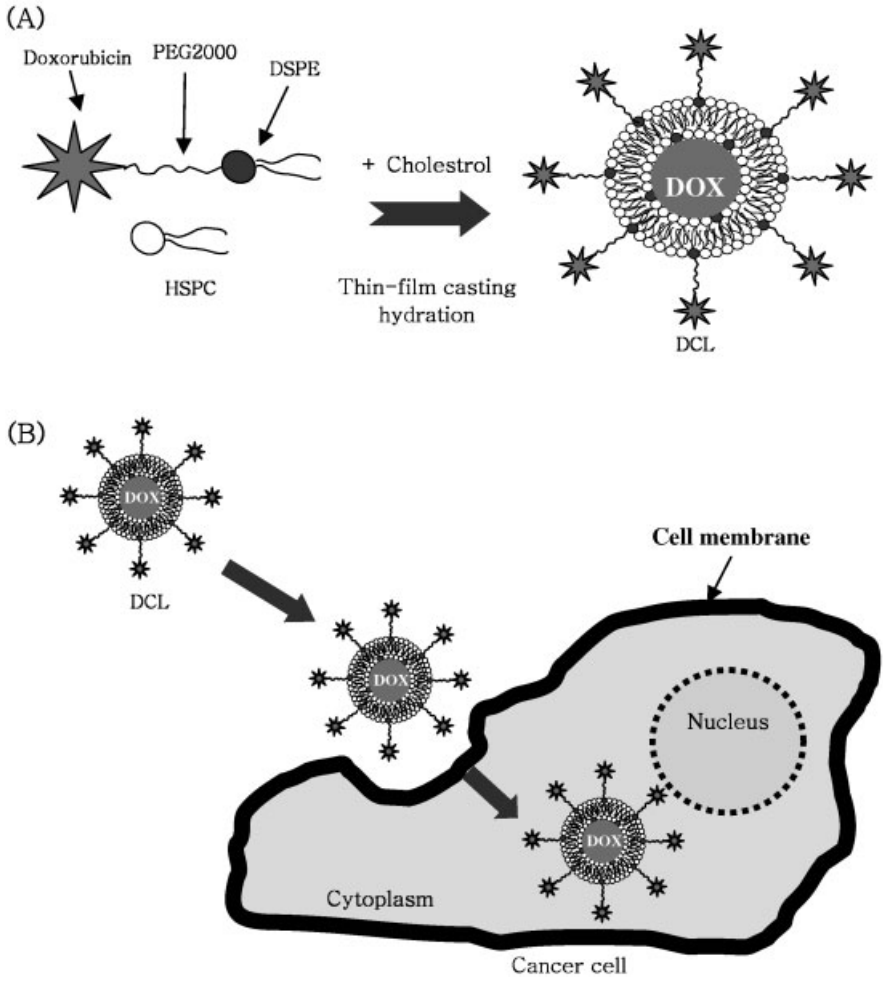

Figure 1.
Schematic illustration of (A) doxorubicin (DOX)-conjugated liposomes (DCL) and (B) cellular uptake of DCL.

Distearoyl-*sn*-Glycero-3-Phosphoethanolamine-*n*-[carboxy(Polyethylene glycol)-2000] (DSPE-PEG2000-COOH) were purchased from Avanti Polar Lipids Inc. (Alabaster, AL, US). DOX was purchased from Boryung Pharm. Co. (Ansan, South Korea). *N*-(3-Dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride (EDC) and *N*-hydroxysuccinimide (NHS) were purchased from Sigma-Aldrich (St. Louis, MO, US). All other chemicals were of analytical grade.

Cell Lines and Mice

B16F10, a murine melanoma cell line, was cultured in DMEM supplemented with 10% (v/v) heat-inactivated fetal bovine serum and 10 μl/ml penicillin streptomycin. The cultures were sustained at 37 °C in a humidified incubator containing 5% CO_2. Male BDF1 mice (6 weeks old) were purchased from OrientBIO Inc. (Seongnam, South Korea). All the procedures involved in the animal studies were performed in accordance with the recommendations of the NIH guideline for the proper use and care of laboratory animals.

Synthesis of DSPE-PEG2000-DOX (DPD)

DPD was synthesized by conjugating DOX to DSPE-PEG2000-COOH covalently. The carboxyl group of DSPE-PEG2000-COOH

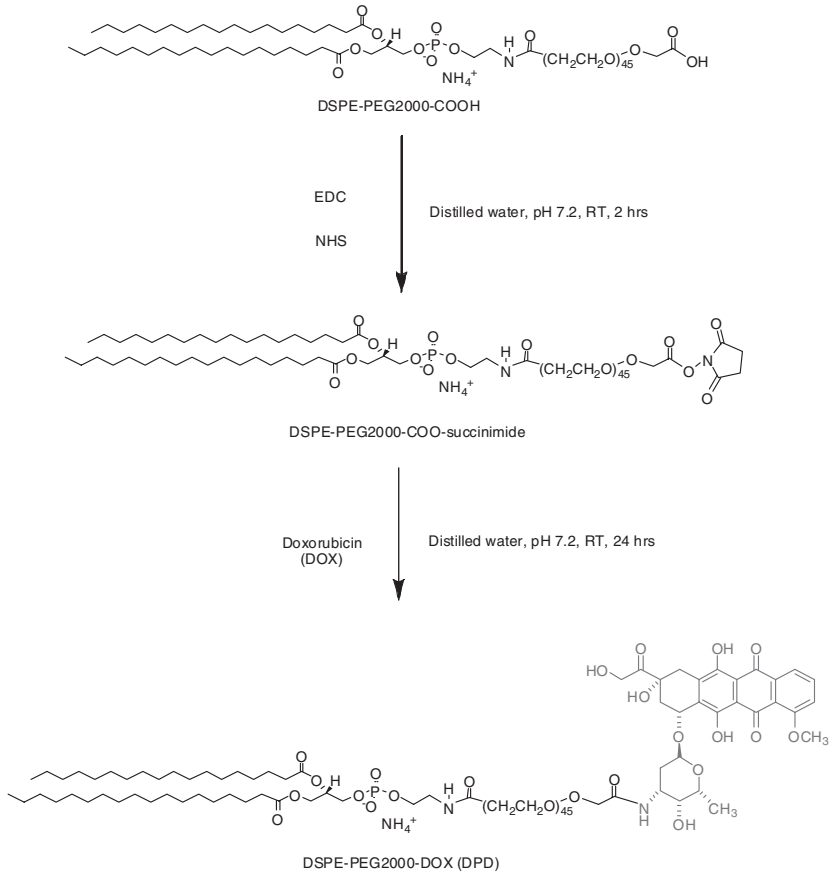

Figure 2.
Synthetic route for DSPE-PEG2000-DOX (DPD). DPD was synthesized by conjugating DOX to DSPE-PEG2000-COOH using EDC and NHS.

was activated by EDC and NHS. Briefly, DSPE-PEG2000-COOH was dissolved in distilled water and activated with EDC and NHS under N_2 atmosphere at room temperature for 2 hrs (mole ratio of DSPE-PEG2000-COOH : EDC : NHS = 1 : 2 : 2). The activated lipid, DSPE-PEG2000- COO-succinimide was reacted with aqueous solution of DOX under N_2 atmosphere at room temperature for 24 hrs (mole ratio of DSPE-PEG2000-COO-succinimide : DOX = 1: 1.5). After the reaction, the solution was dialyzed (MWCO; 1,000) at 4 °C to remove excess EDC, NHS and DOX.

Preparation of Liposomes

DCL was prepared through thin film-hydration method and loading of DOX to the DCL was carried out according to the remote loading method, which uses ammonium sulfate gradient between inside and outside of lipid bilayer of liposomes.[7] Lipid compositions of each liposome was as follows; (1) conventional liposomes; HSPC : CHOL = 9.57:3.19 mg/ml; (2) DCL; HSPC : CHOL : DPD = 9.57 : 3.19 : 3.19 mg/ml. Briefly, the lipids with the above compositions were dissolved in chloroform, dried into a thin film inner layer of round bottomed glass flask using a rotary evaporator and then suspended in 250 mM ammonium sulfate solution. The liposomal solution was extruded through a polycarbonate filter (pore size; 100 nm) using an extruder (Northern Lipid Inc., Canada). The ammonium sulfate that was not

included inside of the liposomes was removed by dialysis (MWCO; 12,000) at 4 °C. The dialyzed liposomal solution and DOX solution (2 mg/ml) were mixed and then incubated at 60 °C for 2 hrs. The DOX-loaded liposomes were dialyzed (MWCO; 12,000) to remove free DOX. The DOX-loaded liposomes were then stored at 4 °C until use.

Characterization of Liposomes

The size of the liposomes was measured by light scattering with a particle size analyzer (ELS-8000, Particle Analyzer, Otsuka, Japan). The concentration of DOX in the liposomes was measured by UV-VIS spectrophotometry at 490 nm (UV-mini, Shimadzu, Japan) and DOX loading efficiency was calculated according to Eq. (1)

$$\text{DOX loading efficiency } (\%) = F_t/F_i \times 100 \quad (1)$$

where F_t is the concentration of DOX loaded in the liposomes after destruction of the liposomes using 20% (v/v) Triton X-100 aqueous solution and F_i is the initial concentration of DOX added to liposome solution. Amount of DOX on the surface of DCL was measured by UV-VIS spectrophotometry at 490 nm without loading DOX to DCL.

Cellular Uptake of Liposomes

The uptake of liposomes to B16F10 cells was evaluated by flow cytometry analysis (1×10^4 cells). Briefly, B16F10 cells were incubated with 15 μg/ml of liposomes in DMEM medium for 2 hrs and then subjected to flow cytometry analysis (Becton-Dickinson FACscan with CELLQuest software, Becton-Dickinson Immune cytometry system, Mountain View, CA).

Antitumor Activity

About 5×10^5 B16F10 murine melanoma cells were inoculated into the right limb armpit of each BDF1 mouse. Ten days after cancer cell inoculation, conventional liposomes, DCL, or free DOX solution was injected intravenously (i.v.) at a single dose of 6 mg DOX/kg body weight. Tumor volume was monitored for up to 24 days after drug administration. Tumor growth was followed by caliper measurements of the tumor. The volume in mm^3 was estimated by the formula; tumor volume $= a(b^2)/2$, where a and b are the tumor length and width respectively in millimeter. Statistical analysis of *in vivo* data was performed using Student's t-test and $P < 0.01$ was considered statistically significant.

Results and Discussion

Synthesis of DSPE-PEG2000-DOX (DPD)

Covalent conjugation of DOX to DSPE-PEG2000-COOH was confirmed by ^{1}H-NMR (500 MHz, Auto Sampler-HRMAS-FT-NMR, Bruker, Switzerland) analysis of DPD. ^{1}H-NMR spectra were obtained for comb polymer solutions in deuterated chloroform. As shown in a Figure 3, the proton peaks of DOX (1, 2 and 3) and DSPE-PEG2000-COOH (4 and 5) were observed at 7.4 ~ 8.2 ppm and 1.2 ~ 3.7 ppm, respectively.

Physical Properties of Liposomes

Physical properties of conventional liposomes and DCL were shown in Table 1. Mean particle size of the conventional liposomes and DCL were 128.1 ± 0.1 and 128.9 ± 1.3 nm, respectively. DOX loading efficiencies of the liposomes were in the range of 75~94%. The amount of DOX on the surface of DCL was 0.049 mg/ml without loading free DOX into DCL.

Cellular Uptake of Liposomes

Flow cytometry analysis of liposomes was performed to compare cellular uptake of conventional liposomes and DCL. Intracellular uptake of DCL was higher than that of conventional liposomes (Figure 4, (A)). Cellular uptake efficiency that was calculated from the area under curve of flow cytometry analysis showed that DOX was transported to cytosol or nucleus of cancer cell by DCL with higher efficiency due to

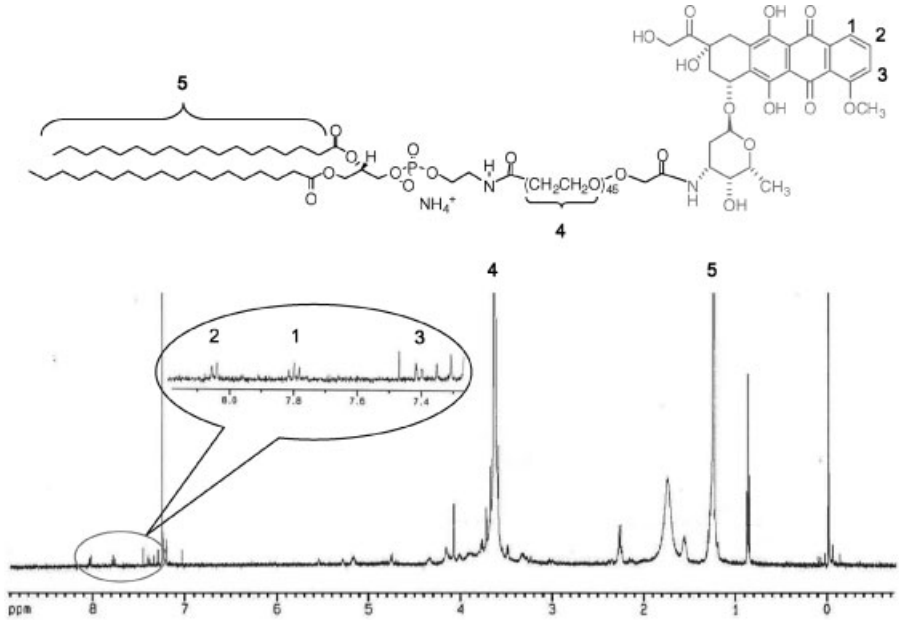

Figure 3.
^{1}H-NMR spectra of DSPE-PEG2000-DOX (DPD). 500 MHz NMR, solvent; $CDCl_3$.

enhancement of membrane binding between DCL and tumor cell membrane.[8,9]

Antitumor Activity

As shown in Figure 5, tumor growth of subcutaneous B16F10 melanoma-bearing mice treated with DCL was more inhibited when compared to those treated with conventional liposomes and free DOX ($P < 0.01$). Therefore, these results indicate that DCL could inhibit tumor growth more efficiently than conventional liposomes. After the 20th day of drug injection, tumor volume of DCL-treated mice increased. However, tumor re-growth of the DCL-treated mice was much more retarded as compared to those of free DOX or conventional liposomes-treated mice. It is considered that more pronounced tumor growth inhibition can be achieved by re-administration of DCL between the 20th day and the 30th day after the first drug injection. All mice survived till the end of this antitumor activity test period even though tumor volume of mice increased. During this test period, no severe sign of toxicity, such as fever, loss of weight, cachexia and myalgia were observed in the mice treated with the lipsomal DOX or free DOX.

Table 1.
Physical properties of conventional liposomes and DCL.

Liposomes (Lipid composition)	Lipid weight ratio	Liposome size (nm)[a)]	DOX loading efficiency (%)[b)]	DOX amount of DCL (mg/ml)
Conventional liposomes (HSPC:CHOL)	3:1	128.1 ± 0.1	94	–
DCL (HSPC:CHOL:DSPE-PEG2000-DOX)	3:1:1	128.9 ± 1.3	75	0.049

[a)] Measured by light scattering.
[b)] Calculated according to Equation (1).

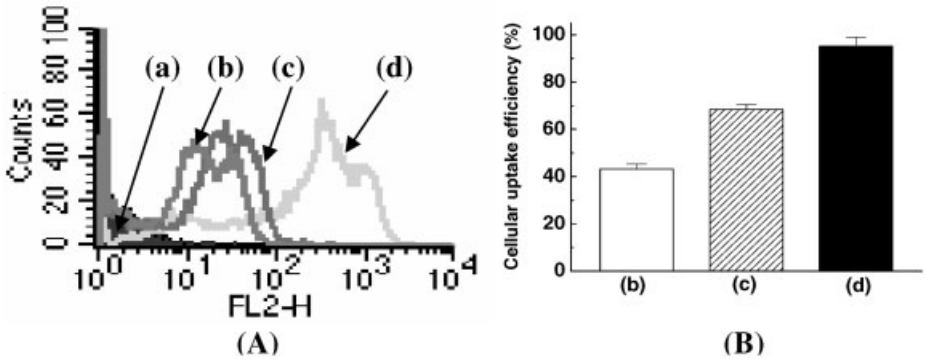

Figure 4.
Flow cytometry analysis for cellular uptake of DOX-loaded liposomes to B16F10 melanoma cells (A) and cellular uptake efficiency of liposomes of free DOX (B); (a) normal cell, (b) conventional liposomes, (c) DCL and (d) free DOX. B16F10 cells of 1×10^4 were incubated with 15 ug/ml of DOX-loaded liposomes in DMEM medium for 2 hrs.

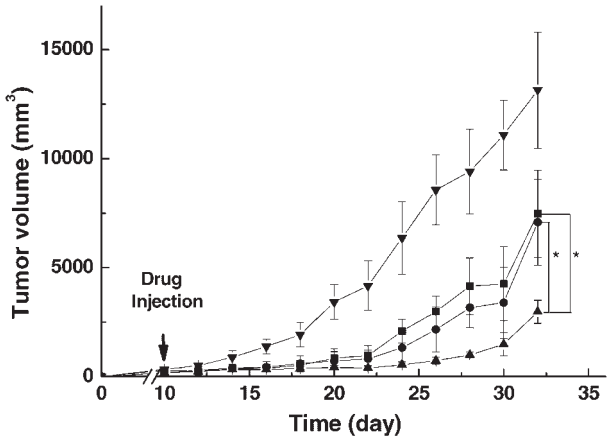

Figure 5.
Tumor growth behavior of B16F10 melanoma-bearing BDF1 mice injected with different liposomal DOX formulations; ▼ = PBS control, ■ = free DOX, ● = conventional liposomes and ▲ = DCL. The arrow indicates the starting day of treatment after the inoculation of the B16F10 tumor cells ($^*P < 0.01$ *vs.* conventional liposomes or free DOX). The data represent the mean ± S.D. (n = 6).

Conclusions

DSPE-PEG2000-DOX (DPD), a neo phospholipid derivative was successfully synthesized and its chemical structure was confirmed by ^{1}H-NMR analysis. DOX-conjugated liposomes (DCL) containing DPD was prepared by using thin-film hydration method and liposome size of DCL was approximately 130 nm. The DCL showed higher intracellular uptake than conventional liposomes and the difference was regarded as increase of passive diffusion of doxorubicin by enhanced membrane binding between DCL and plasma membranes. Antitumor activity of DCL against B16F10 melanoma revealed that DCL could inhibit tumor growth more efficiently than conventional liposomes. These results demonstrated that the enhanced cellular

uptake of DCL may be attributable to DOX mediated endocytosis process. Therefore, the DCL can be used as anticancer drug carrier for the purpose of tumor targeting.

[1] H. D. Han, A. Lee, C. K. Song, T. Hwang, H. Seong, C. O. Lee, B. C. Shin, *Int. J. Pharm.*, **2006**, *313*, 181.
[2] H. S. Yoo, T. G. Park, *J. Control. Release*, **2004**, *96*, 73.
[3] X. Xiong, Y. Huang, W. Lu, X. Zhang, H. Zhang, T. Nagai, Q. Zhang, *J. Control. Rel.*, **2005**, *107*, 262.
[4] E. J. Gabbay, D. Grier, R. E. Fingerle, R. Reimer, R. Levy, S. W. Pearce, W. D. Wilson, *Biochemistry* **1976**, *15*(10), 2062.
[5] H. M. Coley, W. B. Amos, P. R. Twentyman, P. Workman, *Br. J. Cancer*, **1993**, *67*(6), 1316.
[6] D. Marquardt, M. S. Center, *Cancer Res.* **1992**, *52*(11), 3157.
[7] G. Haran, R. Cohen, L. K. Bar, Y. Barenholz, *Biochim. Biophys. Acta.*, **1993**, *1151*, 201.
[8] G. Speelmans, R. W. H. M. Staffhorst, B. Kruijff, F. A. Wolf, *Biochemistry*, **1994**, *33*, 13761.
[9] H. S. Mulder, H. Dekker, H. M. Pinedo, J. Lankelma, *Biochem. Pharmacol.*, **1995**, *50*(7), 967.

