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Comparative Cellular and *In Vivo* Anticancer Studies of Doxorubicin Liposomes Prepared with Different Types of Phospholipids

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ABSTRACT

Liposomes have been widely used to improve drug accumulation in cancer therapy, enhancing anticancer efficacy and reducing side effects. The lipid components of the bilayer membrane define the fluidity of liposomes, which affects drug effectiveness. The present study evaluated the effectiveness of liposomes of differing rigidity in delivering doxorubicin (DOX). In this study, a phospholipid with a high T_m, hydrogenated soy phosphatidylcholine (HSPC), was totally or partially substituted with1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC) or1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE). The liposomes loaded DOX were composed of phosphatidylcholine (HSPC, POPC), both combined and not combined with DOPE, at a molar ratio of total phospholipids: cholesterol and DSPE-$mPEG_{2000}$ were 57:38:5, respectively, and produced using a thin-layer hydration method. The *in vitro* cytotoxicity and *in vivo* antitumor activity of these liposomes was subsequently evaluated. The *in vitro* cytotoxicity assay used murine Lewis lung cancer (LLC) cells and Colon Carcinoma (C26) cells. For *in vivo* evaluation, the sample was intravenously injected into mice's tail veins at a dose of 5 mg DOX per kg of body weight. The results showed that substituting HSPC with POPC resulted in cytotoxicity profiles similar to those of DOX solution on C26 colon cancer and LLC cells. The addition of DOPE to DOX liposomes reduced antitumor activity. It can be concluded that the HSPC substituted with POPC or DOPE reduced liposome rigidity while also lowering *in vivo* antitumor activity.

Keywords: Cancer, liposome, doxorubicin, rapid release, phosphatidylcholine

INTRODUCTION

Efficient drug delivery of chemotherapeutic agents is essential to achieving sufficient antitumor effects (Eldin *et al*., 2015). In cancer therapy, liposomes improve drug accumulation, enhancing anticancer efficacy and reducing side effects (Yingchoncharoen *et al*., 2016). Liposomal drug delivery for tumor therapy represents a promising approach to overcoming the lack of tissue specificity (Lee & Thompson, 2017). However, the drug encapsulated in liposomes is biologically inactive because it needs to be released from liposomes. The effect of slow drug release on the nanoparticles is inadequate drug concentration in the tumor tissue. This is why promising anticancer drugs fail when applied *in vivo* (Seynhaeve *et al*., 2013).

Liposomes are defined as vesicular drug delivery systems composed of cholesterol and

phospholipids (Akbarzadeh *et al*., 2013). Membrane rigidity determines permeability in water during the move toward the intraliposomal phase. It further dissolves the drugs inside, thus creating a gradient concentration for drug diffusion to the extra-liposomal phase (Y. C. Barenholz, 2012; Miatmoko *et al*., 2019, 2021). The physical state of drugs encapsulated within the liposomes restricts their solubility in solvation media before drugs diffuse out from the intraliposomal compartment (Barenholz, 2012; Miatmoko *et al*., 2017).

Intraliposomal drug-trapping agents are primarily employed to maintain the drug's greater physical stability in liposomes. Poly-L-glutamic acid can form stable complexes with weak base drugs. The mechanism forms aggregates or gels inside the liposomes (Drummondy *et al*., 2007; Miatmoko *et al*., 2017). Therefore, their application in drug delivery systems can determine liposomes' ability to load drugs and control drug release. In general, slow drug release from the carriers can be beneficial because of the drug's minimum leakage during systemic distribution *in vivo*. However, slow-release significantly affects drug bioavailability in target tissues (Yang *et al*., 2019).

Doxorubicin (DOX), an anthracycline drug, has been reported to inhibit DNA replication, arrest the cell cycle in G1/G2, and induce apoptosis (McGowan *et al*., 2017). However, DOX can be rapidly cleared, thus limiting its ability to reach tumor tissue in sufficient quantities (Seynhaeve & Hagen, 2017). Certain unwanted side effects such as cardiotoxicity encourage the development of liposome delivery systems (Yang *et al*., 2019). The liposome can accumulate in significant quantities in tumor tissue through the enhanced permeation and retention (EPR) effect, thus improving drug efficacy and safety (Zhao *et al*., 2018).

However, DOX needs to be released from the carrier (Seynhaeve *et al*., 2013). Moreover, according to the report by Seynhaeve *et al*. (2013), the effectiveness of free DOX *in vivo* is higher than that of the liposomes (Seynhaeve *et al*., 2013). DOX can diffuse rapidly into cells and accumulate in the nucleus, although liposome DOX must first be released. The intrinsic stability of DOX in its liposomal form prevents its release even though DOX is already present in the lysosomes.

It has been reported that the encapsulation of Pemetrexed (PMX) in liposomes with fast drug release demonstrated significant potency with regard to *in vivo* tumor growth inhibition in Mesothelioma (MPM) MSTO-211H-bearing mice (Đorđević *et al*., 2014). Meanwhile, PMX encapsulation in rigid liposomes inhibited PMX release from liposomes and reduced its antitumor activity.

The lipid choices for the liposome's bilayer membrane component determine, to a considerable extent, the liposome's rigidity and fluidity. The liposomal components of unsaturated lipids with a low phase transition temperature (T_m) such as 1-palmitoyl-2-oleoyl-sn-glycerophosphocholine (POPC) with the T_m of -2 °C can increase the permeability of liposomes, but reduce their stability. In contrast, saturated phospholipids such as hydrogenated soy phosphatidylcholine (HSPC), which has a T_m of 55 °C, cause the liposome to be more rigid and impermeable (Akbarzadeh *et al*., 2013). In addition, dioleoyl phosphatidylethanolamine (DOPE), a non-bilayer forming lipid, can act as a fusogenic enhancer to improve liposomal fusion with the cellular membranes.

The presence of drugs is also strongly influenced by that of precipitation or drug aggregation in liposomes (Gubernator, 2011). The formation of colloidal aggregates of drugs and polymers in liposomes indicates the release of these drugs (Miatmoko *et al*., 2017)

This study prepared liposomes with total or partial HSPC substitutions using POPC and DOPE. The formulation aims to evaluate the cytotoxicity and antitumor activity of DOX liposomes prepared with different properties or rigidity of phospholipids components. The study involves two DOX-sensitive murine cell lines, *i.e.* Lewis Lung Carcinoma (LLC) cells and Colon Carcinoma (C26) cells, which have been widely used to assess the anticancer efficacy of DOX liposomes (Aloss & Hamar, 2023; Mirhadi *et al*., 2022; Ni *et al*., 2022; Tan *et al*., 2021). These two cell lines can constitute good models for murine solid tumors (Aulino *et al*., 2010; Frajacomo *et al*., 2016; Tan *et al*., 2021). It is reported that DOX promotes high efficacy in suppressing the growth of solid tumors produced in these models (Argenziano *et al*., 2020; Humber *et al*., 2007; J. Lee *et al*., 2023; Rivankar, 2014). The stability of encapsulated DOX within liposomes can greatly affect the anticancer activities of DOX.

MATERIALS AND METHODS

Doxorubicin HCl (DOX) was purchased from LC Laboratories (Woburn, Massachusetts, USA). The lipids, *i.e.* hydrogenated soy phosphatidylcholine (HSPC), 1-palmitoyl-2-oleoyl-sn-glycerophosphocholine (POPC), dioleoyl phosphatidylethanolamine (DOPE), and methoxy-

Table I. Formulation of doxorubicin liposomes prepared in various lipid components (in molars) using PGA in triethylamine (TEA) as an intraliposomal trapping agent.

Component	Formula				
	HSPC-PGA-L	**POPC-DOPE-PGA-L**	**HSPC-DOPE-PGA-L**	**POPC-PGA-L**	**HSPC-AS-L**
HSPC	57	-	34	-	57
POPC	-	34	-	57	-
DOPE	-	23	23	-	-
Cholesterol	38	38	38	38	38
DSPE-$mPEG_{2000}$	5	5	5	5	5
Hydrating Solution	4 mg/mL PGA_{9800} in 0.65 M TEA pH 6.57	4 mg/mL PGA_{9800} in 0.65 M Triethylamine pH 6.57	4 mg/mL PGA_{9800} in 0.65 M Triethylamine pH 6.57	4 mg/mL PGA_{9800} in 0.65 M Triethylamine pH 6.57	0.25 M Ammonium Sulfate pH 5.23

(polyethylene-glycol)-distearoylphosphatidyl-ethanolamine with a mean PEG molecular weight of 2000 (DSPE $mPEG_{2000}$), were sourced from NOF Inc., Tokyo, Japan. Cholesterol and triethylamine (TEA) were the products of Wako Pure Chemical Industries Inc. (Osaka, Japan). Poly-L-glutamic acid with a mean molecular weight of 9800 (PGA_{9800}) was acquired from Sigma (Tokyo, Japan). Other reagents were of the non-technical grade available.

Preparation of doxorubicin liposomes

As previously reported, DOX liposomes were produced using the thin-layer hydration method including the formula (Table I) (Miatmoko *et al*., 2017). Each liposome component was dissolved in chloroform to produce a stock solution. Lipids (HSPC, POPC, DOPE), cholesterol, and DSPE-$mPEG_{2000}$ were subsequently prepared with a molar ratio of 57:38:5 and put into a round bottom flask. The thin layer was obtained by evaporating the organic solvent using a rotary vacuum evaporator at 55 °C. Following the formation of this layer, it was hydrated using 4mg/mL PGA_{9800} solution in 0.65 M TEA pH 6.57 for the PGA-based liposomes (HSPC/POPC/DOPE-PGA-Ls) or 0.25 M ammonium sulfate for HSPC-AS-L. After hydration, the mixtures were sonicated for 30 min.

Liposomes were then eluted through a Sephadex® G50 column with phosphate-buffered saline (PBS) pH 7.4. The DOX was then loaded into liposomes by adding a DOX solution to the suspension of liposomes at a total lipid/DOX = 5/1 ($^{w}/_{w}$). The free DOX was then separated by eluting the mixtures using a Sephadex® G50 column.

***In vitro* cytotoxicity study of Doxorubicin liposomes**

The *in vitro* cytotoxicity assay used murine Lewis lung cancer (LLC) cells and Colon Carcinoma (C26) cells which were subjected to 48 h of drug incubation. First, LLC and C26 cells were cultured in RPMI-1640 media of 10% fetal bovine serum and 100 µg/mL kanamycin sulfate in a humidified atmosphere containing 5% CO_2 at 37 °C. Approximately $1x10^4$ cells were seeded in each well on a 96-well plate, incubated for 24 h, and then treated. To evaluate the cytotoxicity of liposomes, the cells were incubated in media containing different concentrations of DOX liposomes for 48 hours. After treatment, the cell viability was determined using a cell counting kit (CCK-8, Dojindo, Japan) by comparing the sample's absorption in the control group at λ= 450 nm. Finally, the data was analyzed to determine the IC_{50} value.

***In vivo* antitumor activity study of doxorubicin liposomes on LLC-tumor bearing mice.**

This study used 6-week-old female CDF1 mice with a body weight of approximately 20-25 grams purchased from Sankyo Labo (Tokyo, Japan) as subjects to evaluate the *in vivo* antitumor activity of liposomes. The mice were treated according to the Guiding Principles for the Care and Use of Laboratory Animals issued by the Hoshi University Animal Research Committee.

Initially, approximately $1x10^6$ LLC cells were suspended in 100 µL PBS pH 7.4. The cell suspension was then injected subcutaneously into the mice subjects. After obtaining a tumor size of 100-200 mm^3, the sample was intravenously

injected into their tail veins at a dose of 5 mg DOX per kg of body weight. The tumor volume and body weight were measured individually during the study period.

RESULTS AND DISCUSSION

DOX, which belongs to the anthracycline class of antibiotics, is widely used in cancer therapy. DOX is known to have a Log P value of 0.49-1.3, enabling it to penetrate lipids and bind to tissues with ease (Alrushaid *et al*., 2017). However, based on the research findings of Seynhaeve *et al*. (2013), the *in vivo* effectiveness of free DOX is greater than that in the form of liposomes (Seynhaeve *et al*., 2013). The liposome prevents DOX release, even though it is present in the lysosomes, due to the intrinsic stability of DOX liposomes (Seynhaeve *et al*., 2013). In this study, encapsulating DOX into liposomes prepared with various phospholipids resulted in different cytotoxicity profiles in C-26 and LLC cells, leading to high tumor accumulation.

In vitro cytotoxicity study of doxorubicin liposomes

In this study, all liposomes had a similar particle size of approximately 100 nm with a negative zeta potential value of -20 mV. The effects of DOX liposomes on cell viability were evaluated in C26 and LLC cells by incubating the liposomes for 48 hours. The DOX liposome contained PGA_{9800} prepared with HSPC as the phospholipids (HSPC-PGA-L) produced intermediate cytotoxicity in this study with an IC_{50} value of 0.812 µM in LLC cells and 1.918 µM in C26 cells (Figure 1A-B). The lowest cytotoxicity, indicated by the highest cell viability, was observed in DOX liposome prepared with ammonium sulfate (HSPC-AS-L) with an IC_{50} 2.080 µM in LLC cells and IC_{50} of 2.763 µM in C26 cells. However, substituting HSPC with POPC (POPC-PGA-L) and/or DOPE (HSPC/DOPE-PGA-L and POPC/DOPE-PGA-L), partially resulted in similar cytotoxicity to the DOX solution, which ranged from 0.058 to 0.115 µM for LLC cells and 0.397 to 0.857 µM for C26 cells. These findings are aligned with those of our previous study which indicated that the encapsulated DOX inside liposome produces less *in vitro* cytotoxicity against cancer cell lines, while proving more effective in inhibiting *in vivo* tumor growth than the DOX solution (Miatmoko *et al*., 2017; Onishi *et al*., 2017).

The choice of the liposome's bilayer membrane components determines the liposome's rigidity and fluidity (Akbarzadeh *et al*., 2013). Lipids used in this study included HSPC, POPC, and DOPE, all of which have different acyl chain lengths. Unsaturated phospholipids can cause liposomes to be much more permeable and less stable. In contrast, saturated phospholipids with long acyl chains can cause the liposome bilayer membrane to be more rigid and impermeable (Akbarzadeh *et al*., 2013). As a drug-trapping agent inside the liposome, PGA will probably experience ionic interaction with DOX in the intraliposomal phase, producing stable aggregates. A previous study reported that PGA as an intraliposomal trapping agent can increase DOX accumulation in tumors and inhibit tumor growth in mice with the LLC variety (Miatmoko *et al*., 2017)

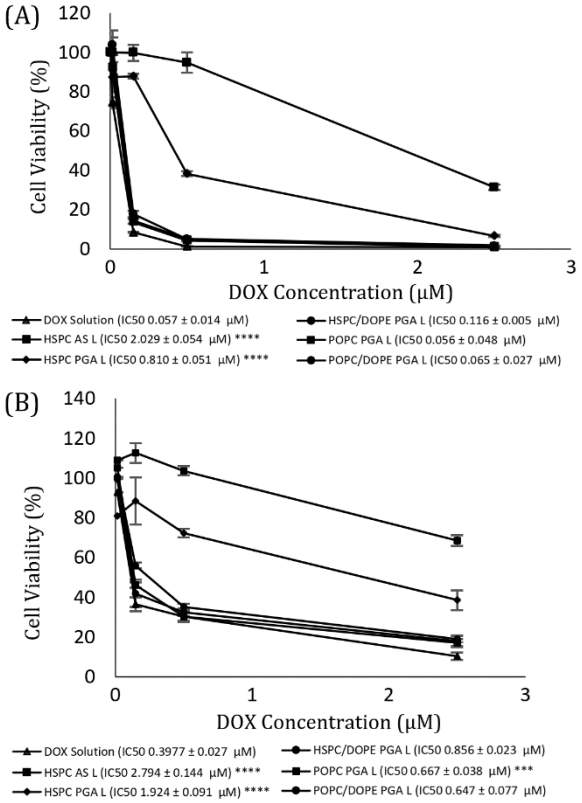

Figure 1. The cytotoxicity of DOX liposomes prepared in various lipids with the addition of PGA_{9800} were evaluated in (A) LLC cells and (B) C26 cells by incubating samples for 48 hours (n=3). **p<0.01, ***p<0.001, ****p<0.0001.

Liposomes were prepared using a fluid-phase phospholipid, POPC (Tm -2 °C), compared to a solid-phase phospholipid, HSPC (Tm 55 °C), which increased the release and permeability of encapsulated DOX by enhancing the fluidity of the liposome membrane (Eldin *et al*., 2015). In addition, the release study in PBS pH 7.4 as the

media at 37 °C showed that the total substitution of HSPC with DOPE and POPC significantly increased drug release from liposomes (Eldin *et al*., 2016). The lipid substitution of liposomes reduces the rigidity of the bilayer membrane. Therefore, the lipid composition of liposomes plays a vital role in drug release. Liposomes with a shorter chain length of lipids and composed of unsaturated acyl chains increase drug release (Drummondy *et al*., 2007). POPC comprises one saturated and one unsaturated acyl chain (Nele *et al*., 2019). The presence of unsaturated chains of POPC causes liposomes to be more fluid with the result that the drug will be released more rapidly compared to liposomes prepared with HSPC that have saturated acyl chains. Previous studies reported that the use of unsaturated phosphatidylcholines as liposomal components resulted in significant higher DOX release than that of liposomes composed of saturated phosphatidylcholines (Drummondy *et al*., 2007).

According to the cytotoxicity assays, substituting HSPC with POPC and DOPE in the PGA-Ls resulted in similar cytotoxicity to free DOX. However, DOX liposomes prepared with HSPC showed intermediate cytotoxicity. In addition, HSPCs prevented rapid drug release from liposomes, resulting in low drug release in tumor cells, thus reducing antitumor effectiveness (Eldin *et al*., 2015). Therefore, the less rigid liposomes cause drugs to be more easily released from liposomes, thereby increasing cytotoxicity and reducing cell viability.

In vivo antitumor efficacy study of doxorubicin liposomes in LLC-tumor bearing mice.

The efficacy of DOX liposomes prepared with ammonium sulfate (AS-L) and PGA_{9800} (HSPC-PGA-L and HSPC-DOPE-PGA-L for inhibiting tumor growth was evaluated in mice injected with LLC tumors. The mice were given a single dose of liposomes equivalent to 5 mg DOX per kg body weight. Negligible body weight changes in the mice occurred during the study (Figure 2A).

The *in vivo* antitumor efficacy of DOX liposomes was evaluated for tumor size in mice with LLC tumors during an 11-day observation period. The HSPC-AS-L showed effective antitumor activity, culminating in the smallest tumor size and percentage tumor growth in comparison with DOX solution(Figure 2B). Meanwhile, partially substituting HSPC with DOPE and using PGA (HSPC/DOPE-PGA-L) resulted in similar antitumor activity as free DOX. These results show that HSPC-AS-L was the most stable liposome among all liposomes and showed the highest antitumor activity.

In vivo study of antitumor activity showed that the lipid substitution of HSPC with DOPE produced low antitumor activity. This may be because the drug leaks out more readily from the liposomes. Thus, DOX encapsulation in fluid liposomes causes low antitumor activity. In contrast, thigh antitumor activity was evident in the HSPC-AS-L formula, where ammonium salts control the stability and release profile of weakly basic drugs (Barenholz, 2007).

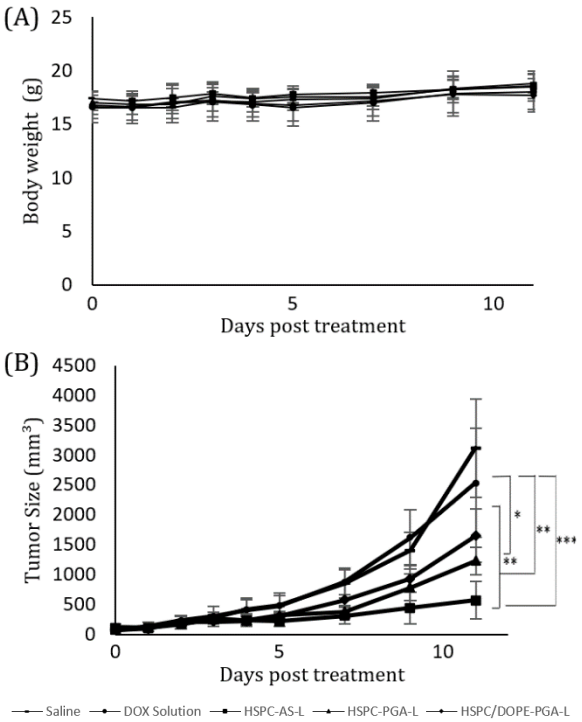

Figure 2. (A) Evaluation of body weight of mice (B) Antitumor activity in terms of tumor size of mice treated with DOX liposomes prepared with various lipid components of the liposomes and the use of intraliposomal trapping agent at an equivalent dose of 5 mg DOX/Kg body weight given as a single injection on day 0 in mice bearing LLC tumors observed for 11 days (n=4-5). *p<0.05, **p<0.01, ***p<0.001.

DOX encapsulation into fluid liposomes enhances DOX release thus increasing the cytotoxicity of DOX liposomes which is characterized by reduced cell viability. The more fluid the formulation of DOX liposomes, the greater the drug release and *in vitro* cytotoxicity. However,

this also leads to fewer efficacies for *in vivo* antitumor studies. This is probably due to the instability of DOX in liposomes. More fluid formulation of liposomes causes instability of DOX encapsulation which, in turn, induces drug release from liposomes during systemic circulation. These results are contradictive with the PMX liposome, in which the more fluid the liposomal membrane, the higher the antitumor efficacy potentially achieved. Furthermore, the high permeability of DOX, as represented by its Log P value of 0.49-1.3 (Alrushaid *et al*., 2017) produces easy DOX molecule transport to and from the intraliposomal phase, thus causing considerable drug leakage in fluidic liposomes.

On the other hand, PMX is a molecule with low biological permeability which is actively transported by the cells (Liang *et al*., 2019; Pangeni *et al*., 2018). The high concentration of drug in the bloodstream would enable cellular transport which is extensive, although still limited compared to highly diffused DOX molecules. Therefore, high drug leakage would promote rapid cellular uptake and *in vivo* efficacy of PMX.

The design of liposome formulations as carriers is the primary key to determining the efficacy of drug delivery to tumor tissue as a therapeutic target. However, an in-depth study of the specific physicochemical characteristics of each active ingredient is required where the encapsulation of DOX into liposomes requires a rigid and stable phospholipid-liposome formulation. Further studies are necessary to examine the detailed mechanism of DOX released from liposomes, especially in biological environments to enable their being reviewed together with existing reports. Hence, the study of DOX formulation and its delivery in cancer therapy will be thoroughly reviewed so that it can be used as a truly effective tool for drug delivery design.

CONCLUSION

It can be concluded that total or partial lipid substitution of HSPC with POPC or DOPE in PGA-Ls yielded similar *in vitro* cytotoxicities to free DOX. However, substituting HSPC with DOPE did not increase the antitumor activity of HSPC-PGA-L. Therefore, the encapsulation of DOX in liposome formulation containing low T_m phospholipid, i.e., POPC and fusogenic lipid DOPE, had no benefits in terms of enhancing *in vivo* the antitumor efficacy of DOX.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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