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Phospholipids as Multidrug Resistance Modulators of the Transport of Epirubicin in Human Intestinal Epithelial Caco-2 Cell Layers and Everted Gut Sacs of Rats

*Yu-Li Lo**

Department of Pharmacy, Chia Nan University of Pharmacy and Science, Tainan Hsien, Taiwan 717, R.O.C.

ABSTRACT. Phospholipids have been increasingly used as carriers for the delivery of a variety of drugs. Studies using cancer chemotherapeutic agents such as epirubicin encapsulated in liposomes, which are made of phospholipids and other ingredients, have generally shown reduced toxicity and enhanced therapeutic efficacy. The recent investigation of the role of P-glycoprotein (P-gp) in phospholipid translocation has opened a new area of research on the possible use of phospholipids as multidrug resistance (MDR) modulators. This study investigated the effects of liposomal encapsulation, empty liposome pretreatment, or free lipid pretreatment on the uptake and transport of epirubicin in the human colon adenocarcinoma cell line Caco-2 and in everted gut sacs of rat jejunum and ileum. Epirubicin uptake experiments, using a flow cytometer, showed that both liposomal encapsulation and empty liposome pretreatment increased the intracellular accumulation of epirubicin in Caco-2 cells significantly. These two treatments substantially increased apical-to-basolateral absorption of epirubicin across Caco-2 monolayers and markedly improved mucosal-to-serosal absorption of epirubicin in rat jejunum and ileum. Enhancement also was observed with both liposome encapsulation and empty liposome pretreatment in the reduction of basolateral-to-apical efflux of epirubicin across Caco-2 monolayers. However, because diffusion of free dipalmitoyl phosphatidylcholine (DPPC) or dipalmitoyl phosphatidylethanolamine (DPPE) lipids across the cell membrane is very slow, these free lipids showed marginal effects on absorption and/or secretion of epirubicin in both Caco-2 cells and rat gut sacs. The study suggests that inhibition of P-gp or other transporter proteins located in the intestines may be partially involved in the reduction of epirubicin efflux. In conclusion, the therapeutic efficacy of epirubicin may be improved by using phospholipids as excipients and MDR modulators in the formulations. Liposomal formulations may have important applications to circumvent drug resistance in cancer chemotherapy. BIOCHEM PHARMACOL **60**;9:1381–1390, 2000. © 2000 Elsevier Science Inc.

KEY WORDS. epirubicin; phospholipids; liposomes; Caco-2; rat intestine; P-glycoprotein

The recent investigation of the involvement of P-gp† in phospholipid translocation has opened a new area for research on the role of P-gp in lipid regulation. Phospholipids are the major lipids in the plasma membrane of mammalian cells. They have been found to be involved in signal transduction in cells. In addition, phospholipids have been used increasingly as carriers for the delivery of a variety of drugs. Studies using cancer chemotherapeutic agents such as epirubicin encapsulated in liposomes made of phospholipid mixtures have shown reduced toxicity and enhanced therapeutic efficacy [1].

MDR, mediated by the increased expression of energy-dependent drug efflux pumps, such as P-gp, appears to be a major obstacle to the successful treatment of clinical tumors [2]. P-gp is a member of a multigene family with two homologues in humans and three in mice. P-gp homologues that transport drugs, such as human MDR1 and mouse mdr3 and mdr1, are designated as Class I or Class II [3]. P-gp homologues that do not transport cytotoxic agents, e.g. human MDR3 and mouse mdr2, belong to Class III P-gp [4]. MDR1 P-gp is thought to function as an ATP-dependent drug transporter, which reduces the intracellular concentration of cytotoxic drugs of the MDR spectrum [5]. The MDR1 P-gp is expressed in the apical or luminal regions of several natural epithelial and endothelial cell types, especially in the gastrointestinal tract and in liver biliary hepatocytes [2]. It also occurs at the surface of many tumor cells [5]. Class III P-gp has been shown to function as

* Corresponding author: Yu-Li Lo, Ph.D., Department of Pharmacy, Chia Nan University of Pharmacy and Science, 60 Erh-Jen Road, Sec. 1, Jen-Te Hsiang, Tainan Hsien, Taiwan 717. Tel. (886) 6-266-4911, Ext. 202; FAX (886) 6-266-6411; E-mail: yulilo@ms17.hinet.net

† *Abbreviations*: P-gp, P-glycoprotein; MDR, multidrug resistant/resistance; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; DPPC, dipalmitoyl phosphatidylcholine; DPPE, dipalmitoyl phosphatidylethanolamine; PA, phosphatidic acid; and DMEM, Dulbecco's modified Eagle's medium.

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a phospholipid translocase [4, 6–9]. No drug-pumping activity has been demonstrated thus far for Class III P-gp, which is present in high concentrations in the bile canalicular membrane.

Studies have shown that Class III P-gp specifically translocates PC toward the outer bilayer leaflet of the plasma membrane [4, 6, 7, 9]. Evidence also has suggested that MDR1 P-gp translocates PC, PE, sphingomyelin, and glucosylceramide across the plasma membrane [7]. It also has been found that PC and PE but not PS behave as substrates for human MDR1 P-gp in two multidrug-resistant cell lines expressing human MDR1 [8]. In our previous study, we proposed that the effect of BSA on the transport of vinblastine or epirubicin may be mediated through its effect on the extraction of phospholipids, e.g. PC and PE, into the cell surface. This results in fewer P-gp substrates and more P-gp available for pumping epirubicin or vinblastine out of Caco-2 cells [10, *]. Besides confirming a well-known role of MDR1 P-gp as a multidrug transporter, these studies explored its physiological role as a flippase in translocating lipids of broad specificity across the plasma membrane [4, 6–10].

There are overlapping physicochemical characteristics between hydrophobic cationic drugs, such as epirubicin, which are transported by Class I P-gp, and certain zwitterionic phospholipids, such as PC and PE, which are transported by Class I and/or Class III P-gp. Thus, modulation of P-gp by phospholipids through substrate competition or other mechanisms may antagonize MDR and increase the cytotoxicity of epirubicin.

The Caco-2 cell line expresses many characteristics of differentiated cells of the normal human small intestine. It is increasingly used as an *in vitro* model for the study of drug transport across the intestinal epithelium. P-gp is present in the apical membrane of Caco-2 cells, where it is responsible for the polarized transport of various drugs, and possibly phospholipids, across cell monolayers.

In this study, epirubicin was chosen as a model anticancer drug. Epirubicin has an anti-tumor activity spectrum comparable to that of doxorubicin, but is better tolerated and less myelo- and cardiotoxic than doxorubicin [11]. Epimerization of the 4′-OH group of the amine sugar moiety of epirubicin results in less ionization, higher lipophilicity, and greater cellular uptake [12].

In this study, we demonstrated the effects of various phospholipid formulations on the intracellular accumulation of epirubicin in Caco-2 cells using a flow cytometer. These formulations included the following six preparations: epirubicin encapsulated in DPPC or DPPE liposomes, pretreated with empty DPPC or DPPE liposomes, and pretreated with free DPPC or DPPE. Furthermore, the functional involvement of P-gp in this process was verified by the addition of verapamil, one conventional P-gp reversing agent. With Caco-2 monolayers grown in Transwell filters as a model, the effects of phospholipid formulations on both apical-to-basolateral and basolateral-to-apical transport of epirubicin were studied. The apparent permeability coefficients (P_{app}) of epirubicin were calculated in both the donor and receiver compartments according to the equation $P_{app} = (dQ/dt)/(A \cdot C_0)$, where dQ/dt is the drug permeation rate, A is the cross-sectional area, and C_0 is the initial epirubicin concentration in the donor compartment at t = 0 [13]. In addition, the effects of these formulations on the intestinal absorption of epirubicin were investigated in everted gut sacs prepared from rat jejunum and ileum.

* Lo YL, Hsu CY and Huang JD, Manuscript in preparation.

MATERIALS AND METHODS

Chemicals and Animals

Epirubicin was purchased from Pharmacia & Upjohn. Verapamil was obtained from the Sigma Chemical Co. DPPC (>99% purity) and DPPE (>99.5% purity) were purchased from Avanti Polar Lipids Inc. All cell culture media and reagents were purchased from GIBCO Inc. Most of the other chemical reagents were purchased from Merck. Male Sprague–Dawley rats bred and housed in the animal center of National Cheng-Kung University Medical College were used. Tyrode's solution was prepared by dissolving 24 g of NaCl (137 mM), 3 g of dextrose (5.6 mM), 3 g of $NaHCO_3$ (12 mM), 6 mL of 10% KCl (2.7 mM), 7.8 mL of 10% $MgSO_4 \cdot 7H_2O$ (1.1 mM), 3.9 mL of 5% $NaH_2PO_4 \cdot 2H_2O$ (0.42 mM), and 5.4 mL of 1 M $CaCl_2$ (1.8 mM) in 3 L of water.

Cell Culture

Caco-2 cells (obtained from the American Type Culture Collection) were maintained at 37° in DMEM supplemented with 15% fetal bovine serum, 1% nonessential amino acids, 2 mM L-glutamine (GIBCO Inc.), and 10,000 U/mL of penicillin/streptomycin (GIBCO Inc.), in an atmosphere of 5% CO_2 and 90% relative humidity.

Liposome Preparation

DPPC or DPPE was dissolved in a minimal amount of absolute ethanol. Epirubicin was dissolved in serum-free DMEM for flow cytometric and transport studies. In the everted sac study, epirubicin was dissolved in Tyrode's solution. The lipid/ethanol mixture was injected rapidly into epirubicin solution, which was warmed to 45° and stirred magnetically. The solvent in this mixture then was removed by evaporation. The lipid to epirubicin ratio was maintained at 1 μmol:2 μg. Empty liposomes for control experiments were prepared in the same way without the addition of epirubicin. Free lipids were prepared by dispersing DPPC or DPPE into serum-free DMEM or Tyrode's solution and vortexing for 2 min.

Epirubicin Encapsulation Efficiency Determination

The epirubicin-containing liposomes were separated from free drugs by filtering the liposome dispersion under vacuum (5 mm Hg) through a 0.025-μm filter (Millipore Co.). The liposomes then were washed with buffer to remove the free drugs completely. The free epirubicin concentration was analyzed quantitatively by a Hitachi F2000 Fluorescence Spectrophotometer. Epirubicin encapsulation efficiency was calculated as the ratio of the amount of epirubicin in liposomes to the total amount of epirubicin in the aqueous suspension. Each experiment was performed in triplicate.

Flow Cytometric Studies

Measurements of intracellular epirubicin fluorescence were made as described before [14, 15]. Cells ($3 \times 10^5/cm^2$) were seeded into 24-well plates and incubated for 21 days. Cells were washed twice with PBS and incubated for 30 min at 37° in serum-free medium containing empty DPPC liposomes, empty DPPE liposomes, free DPPC, or free DPPE at a concentration of 0.5 mM, as well as medium for the epirubicin liposome treatments. When evaluating the effect of verapamil on epirubicin accumulation, these pretreatments also included 10 μM verapamil. At time zero, a 1 μg/mL concentration of epirubicin or of epirubicin liposomes was added to the medium. Epirubicin or epirubicin liposomes were removed after incubation for 30, 60, 90, 120, 150, or 180 min. The cells were washed with ice-cold PBS and then rinsed with 0.25% trypsin in a 1 mM EDTA solution. The cells were collected into centrifuge tubes, centrifuged for 10 min at 1000 *g*, and then resuspended in PBS. Flow cytometric analysis was conducted on a FACSort flow cytometer (Becton Dickinson) equipped with an argon ion laser (Spectra Physics), which was operated at 488 nm and 15 mW. Red epirubicin fluorescence was collected through a 585/42 nm band pass filter. Data acquisition and analysis were performed with Lysis II software (Becton Dickinson). Forward and side scatter signals were collected using linear scales, and fluorescence signals were collected on a logarithmic scale. At least 10,000 cells were analyzed in each sample. Each experiment was repeated six times.

Transport Studies

TRANSPORT OF EPIRUBICIN IN THE APICAL-TO-BASOLATERAL (A→B) DIRECTION. The Caco-2 cells were seeded into 0.45-μm-pore Transwell inserts at 80,000 cells/cm^2 and grown for 21 days. Cell monolayers were used when the transepithelial electrical resistance exceeded 300 $\Omega \cdot cm^2$ [16]. The transepithelial electrical resistances of the Caco-2 cell monolayers were measured periodically using the MILLICELL electrical resistance system (Millipore Co.) to monitor cell layer confluence and the integrity of tight junctions. Then the transport studies were performed as modified in previous studies [15, 17]. The cells were rinsed twice with PBS and allowed to incubate at 37° in the apical (donor) compartments for 30 min with 0.4 mL of serum-free DMEM containing empty DPPC liposomes, empty DPPE liposomes, free DPPC, free DPPE, or plain medium in the case of epirubicin liposome treatment. Serum-free DMEM (1.5 mL) was placed in the basolateral (receiver) compartments. To initiate the experiment, the donor solution, serum-free DMEM containing 100 μg/mL of epirubicin or epirubicin liposomes, was added followed by incubation in the dark at 37°. Samples (0.2 mL) from the receiver compartments were taken at 10, 20, 30, 60, 120, and 180 min. The receiver compartments were replenished with fresh serum-free DMEM after each sampling. Each experiment was repeated four times.

TRANSPORT OF EPIRUBICIN IN THE BASOLATERAL-TO-APICAL (B→A) DIRECTION. Serum-free DMEM containing empty liposomes, free lipids, or neither addition was added to the basolateral compartments of the Transwell in a volume of 1.5 mL for 30 min, as previously described [15, 17]. Epirubicin or a preparation of epirubicin liposomes (100 μg/mL) then was added to the basolateral (donor) solution, and mixed with gentle agitation. Serum-free DMEM (0.4 mL) was added to the apical compartments to initiate transport. Samples (0.2 mL) from the apical side were removed at 10, 20, 30, 60, 120, and 180 min. The receiver compartments were replenished with fresh serum-free DMEM after each sampling. Each experiment was repeated four times.

Everted Sacs of Rat Jejunum and Ileum

Everted sacs of rat jejunum and ileum were prepared using a method described previously [15, 18]. Male Sprague–Dawley (SD) rats weighing about 300 g were deprived of food for 1 day, and provided with only double-distilled water before the experiments. The rats were anesthetized with ether before the experiment. The jejunum and distal ileum of the rat intestines (approximately 25 cm each) were taken, and the underlying muscularis was removed prior to mounting in Tyrode's solution. Two sacs were everted and filled with 3 mL of Tyrode's solution, and then placed in 50 mL of Tyrode's solution for 30 min. The 50-mL solution contained empty DPPC liposomes, empty DPPE liposomes, free DPPC, free DPPE, or only Tyrode's solution in the case of epirubicin liposome treatment. Then 100 μg/mL concentrations of epirubicin or epirubicin liposomes were added at 37°. In each study, 200 μL of the solution inside the sacs was taken every 10 min for 60 min. Each experiment was performed in triplicate.

Analysis of Concentration of Epirubicin by HPLC

The analytic method for epirubicin was modified from a previous report [19]. Daunorubicin was used as an internal standard. The HPLC system consisted of a Hitachi L7100 pump equipped with a satellite 710B WISP automated

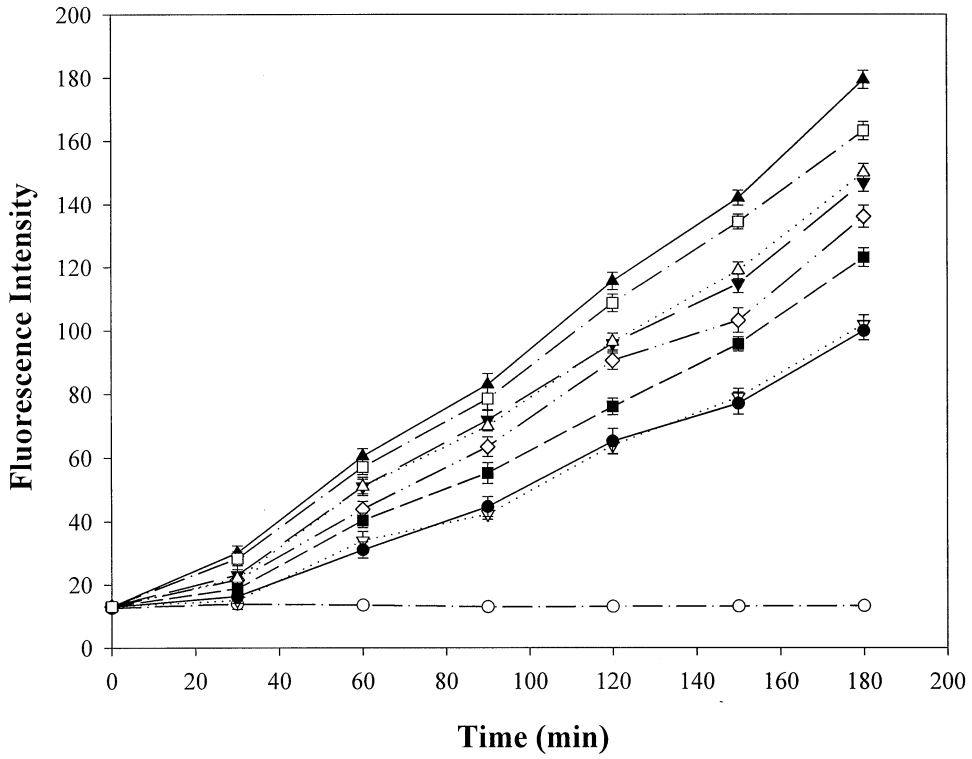

FIG. 1. Time course of the intracellular accumulation of fluorescent epirubicin treated with different DPPC formulations in Caco-2 cells. Cells were pretreated with serum-free medium containing 0.5 mM empty DPPC liposomes or free DPPC in the presence or absence of 10 μM verapamil for 30 min. In the cases of epirubicin control or epirubicin–DPPC liposome treatment, serum-free medium and/or verapamil were added for 30 min. At time zero, a 1 μg/mL concentration of epirubicin or of epirubicin liposomes was added to the medium. Epirubicin or epirubicin liposomes were removed after incubation for 30, 60, 90, 120, 150, and 180 min. The cells then were subjected to flow cytometric measurements. Each point represents the mean ± SD of six determinations. Key: (○) cell control; (▽) epirubicin pretreated with free DPPC; (●) epirubicin control; (▼) epirubicin pretreated with verapamil; (◇) epirubicin encapsulated in DPPC liposomes; (■) epirubicin pretreated with empty DPPC liposomes; (▲) epirubicin encapsulated in DPPC liposomes and pretreated with verapamil; (Δ) epirubicin pretreated with free DPPC plus verapamil; and (□) epirubicin pretreated with empty DPPC liposomes plus verapamil.

injector (Millipore Co.), a 5-μm Lichrospher column (25-cm length, 4-mm inside diameter, Merck), and a Spectroflow 757 UV detector. The mobile phase included methanol:water (75:25, v/v) plus 0.5% acetic acid and 2.5 mM sodium heptanesulfonic acid, run at a flow rate of 1.2 mL/min. The detection wavelength was 254 nm. The ratio of epirubicin to daunorubicin by peak height was compared with the calibration curve for quantitation.

Data Analysis

Results are given as means ± SD. Statistical comparisons were made using Student's *t*-test; $P < 0.05$ was considered significantly different and $P > 0.5$ not significantly different from the control.

RESULTS

Determination of Efficiency of Epirubicin Encapsulation

The encapsulation efficiency (%) of epirubicin into DPPC or DPPE liposomes was 84.6 ± 1.2 and 86.5 ± 0.9, respectively (N = 3).

Flow Cytometric Studies

By direct measurement of intracellular anthracycline fluorescence, a flow cytometer can be used to study the intracellular concentration of epirubicin in Caco-2 cells. As shown in Fig. 1, fluorescence intensity increased as the epirubicin treatment period increased from 0 to 180 min, indicating that the intracellular accumulation of epirubicin increased as time increased. Liposomal encapsulation or empty liposome pretreatment significantly increased the intracellular accumulation of epirubicin in Caco-2 cells, with a better enhancement factor for the former treatment ($P < 0.001$, N = 6; data for DPPE not shown). The effect of free lipids, e.g. DPPC or DPPE, on epirubicin uptake was marginal ($P > 0.5$, N = 6).

The functional involvement of P-gp in epirubicin transport was verified by pretreatment with verapamil, a typical P-gp substrate. The effect of verapamil on the modulation of epirubicin accumulation in the presence or absence of various lipid treatments was evaluated. As displayed in Fig. 1, verapamil increased the intracellular accumulation of epirubicin significantly, verifying the MDR-reversing effect

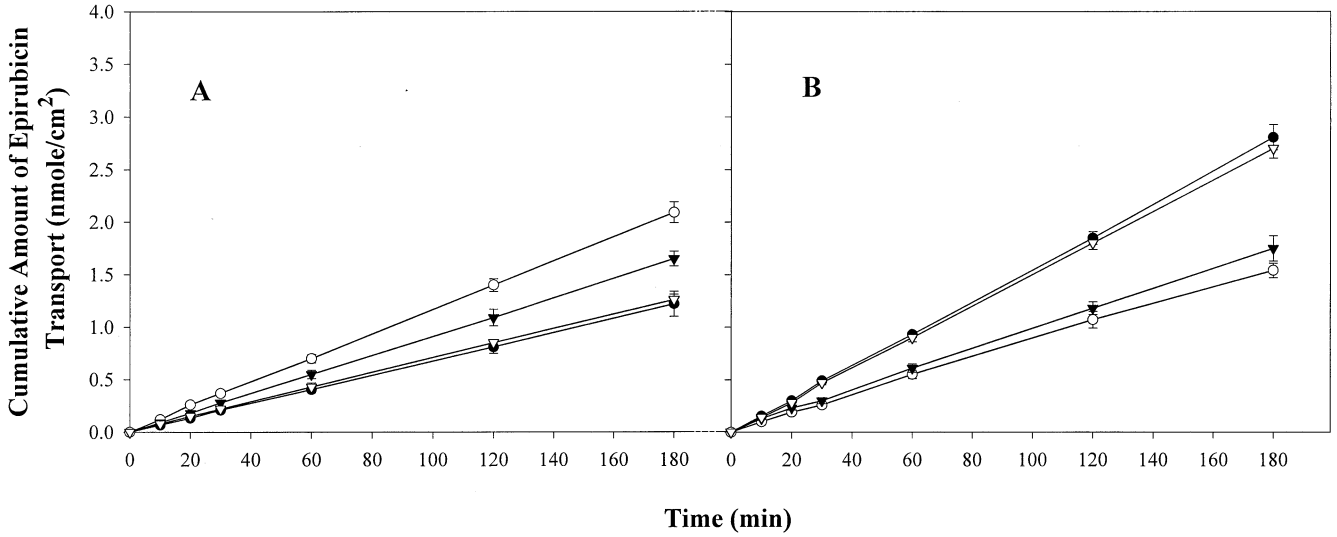

FIG. 2. Transepithelial fluxes of epirubicin across Caco-2 cell monolayers at 37° in the (A) absorptive (apical-to-basolateral; a→b) and (B) secretory (basolateral-to-apical; b→a) directions, plotted against time of incubation, in the presence or absence of various DPPC treatments. Each point represents the mean ± SD of four determinations. Key: (●) epirubicin control; (○) epirubicin encapsulated in DPPC liposomes; (▼) epirubicin pretreated with empty DPPC liposomes; and (▽) epirubicin pretreated with free DPPC.

of verapamil (N = 6, $P < 0.001$). Pretreatment with verapamil in the presence of liposomal epirubicin further increased the epirubicin fluorescence more than did the treatments with verapamil or liposomal encapsulation only (N = 6, $P < 0.01$). Meanwhile, pretreatment with verapamil in the presence of empty DPPC or DPPE liposomes reflected moderate further enhancement compared with the effects of pretreatment with verapamil or empty liposomes only (N = 6, $P < 0.05$). However, pretreatment with verapamil in the presence of free DPPC or DPPE showed only slight additional enhancement of epirubicin uptake (N = 6, $P < 0.1$).

Transport of Epirubicin in the Apical-to-Basolateral (A→B) Direction or Basolateral-to-Apical (B→A) Direction

Figures 2 and 3 show the transepithelial fluxes of 100 μg/mL of epirubicin across Caco-2 cell monolayers at 37° in the absorptive (apical-to-basolateral; a→b) and secretory (basolateral-to-apical; b→a) directions versus time of incubation in the presence or absence of various lipid treatments. The flux of epirubicin in the basolateral-to-apical direction was 2.29-fold of the flux in the apical-to-basolateral direction. Net flux of epirubicin, therefore, was ob-

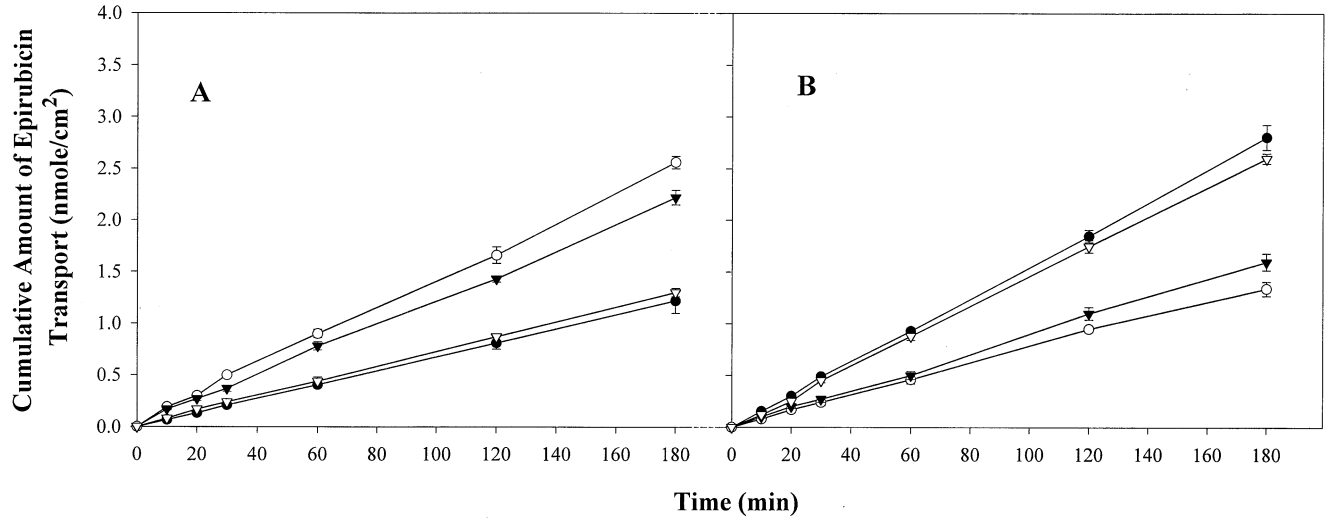

FIG. 3. Transepithelial fluxes of epirubicin across Caco-2 cell monolayers at 37° in the (A) absorptive (a→b) and (B) secretory (b→a) directions, plotted against time of incubation, in the presence or absence of various DPPE treatments. Each point represents the mean ± SD of four determinations. Key: (●) epirubicin control; (○) epirubicin encapsulated in DPPE liposomes; (▼) epirubicin pretreated with empty DPPE liposomes; and (▽) epirubicin pretreated with free DPPE.

TABLE 1. Effects of liposomal encapsulation, empty liposome pretreatment, or free lipid pretreatment on apparent permeability coefficients (P_{app}) of epirubicin in Caco-2 cells*

Treatments	P_{app} epirubicin (cm/sec) $\times 10^{-6}$	†		†	
	$P_{app, a \rightarrow b}$		$P_{app, b \rightarrow a}$		$P_{app, net}$‡
Epirubicin control	0.61 ± 0.04		1.41 ± 0.03		0.80 ± 0.04
Epirubicin encapsulated in DPPC liposomes	1.05 ± 0.05§	↑ 72%	0.78 ± 0.04§	↓ 45%	−0.27 ± 0.05§
Epirubicin pretreated with empty DPPC liposomes	0.83 ± 0.05§	↑ 36%	0.87 ± 0.04§	↓ 38%	0.04 ± 0.05§
Epirubicin pretreated with free DPPC	0.63 ± 0.05§	NS‖	1.36 ± 0.04§	NS	0.73 ± 0.05
Epirubicin encapsulated in DPPE liposomes	1.26 ± 0.07§	↑ 107%	0.68 ± 0.02§	↓ 52%	−0.58 ± 0.07§
Epirubicin pretreated with empty DPPE liposomes	1.10 ± 0.06§	↑ 80%	0.81 ± 0.03§	↓ 43%	−0.29 ± 0.06§
Epirubicin pretreated with free DPPE	0.65 ± 0.05	NS	1.32 ± 0.04§	↓ 6%	0.67 ± 0.05§

*Data representd the means ± SD of four independent experiments.

†Percentage increase or decrease was calculated as $[(\tau^2 - \tau_1)/\tau_1] \times 100$, where τ_1 is the P_{app} of the epirubicin control and τ_2 is the P_{app} of epirubicin after application of various lipid treatments.

‡$P_{app, net}$ values were calculated as $P_{app, net} = P_{app, b \rightarrow a} - P_{app, a \rightarrow b}$. Positive values represent net mucosal secretion of epirubicin, and negative values indicate net mucosal absorption of epirubicin.

§Significantly different from the control by Student's *t*-test ($P < 0.05$).

‖Not significant.

served in the secretory direction in Caco-2 cells. The polarized transport of epirubicin correlated well with the characteristics of P-gp substrates. As illustrated in Table 1, the apparent permeability coefficient (P_{app}) of 100 μg epirubicin/mL in the absorptive direction was an order of magnitude less than that observed in the secretory direction. Net transport of epirubicin was thus in the secretory direction. The DPPC liposomal encapsulation shifted the net transepithelial transport of epirubicin from the secretory direction to the absorptive direction by both an increase in a→b P_{app} (↑ 72%) and a reduction of b→a P_{app} (↓ 45%). Therefore, epirubicin–DPPC liposomes showed a much more pronounced enhancement of epirubicin absorption than reduction of epirubicin efflux. Pretreatment with empty DPPC liposomes demonstrated a moderate enhancing effect on epirubicin absorption. This treatment reduced the net secretory transport of epirubicin across the Caco-2 cells. The net P_{app} was given by both an increase in a→b P_{app} (↑ 36%) and a reduction of b→a P_{app} (↓ 38%). The pretreatment of free DPPC showed marginal reduction of epirubicin efflux ($P > 0.5$, $N = 4$).

As shown in Table 1, the DPPE liposomal encapsulation further shifted the net transepithelial transport of epirubicin from the secretory direction to the absorptive direction; net P_{app} was -0.58×10^{-6} cm/sec as a result of both an increase in a→b P_{app} (↑ 107%) and a reduction of b→a P_{app} (↓ 52%). Pretreatment with empty DPPE liposomes also shifted the epirubicin transport in the absorptive direction. The net P_{app} was obtained by both an increase in a→b P_{app} (↑ 80%) and a reduction of b→a P_{app} (↓ 43%). Pretreatment with free DPPE lipids slightly reduced the secretory transport of epirubicin across Caco-2 cells ($P < 0.5$, $N = 4$).

Everted Sacs of Rat Jejunum and Ileum

As illustrated in Fig. 4, epirubicin was transported from the mucosal side (bulk solution) to the serosal side (inside sac)

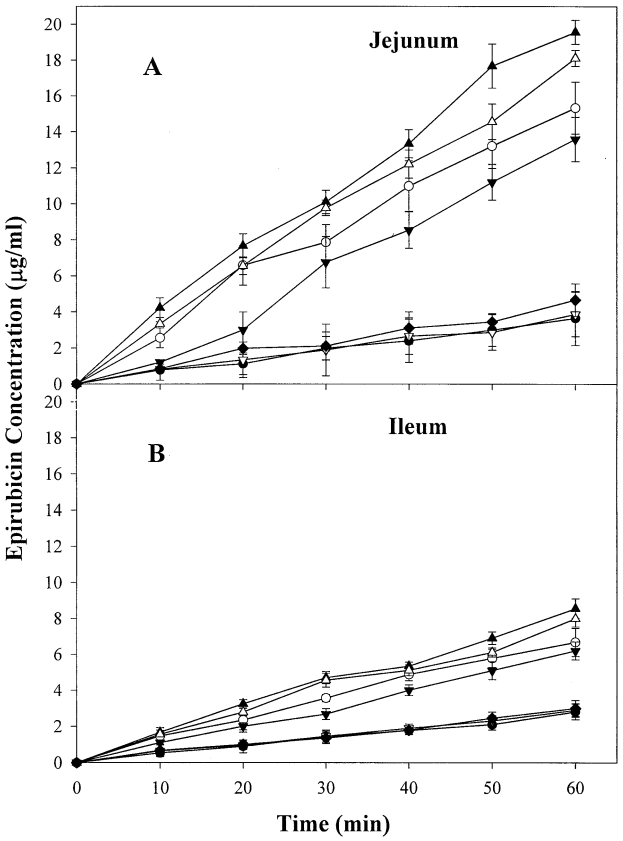

FIG. 4. Time profile of epirubicin concentrations inside everted sacs of jejunum (A) or ileum (B) of rats in the presence or absence of various DPPC or DPPE treatments. Each point represents the mean ± SD of triplicate determinations. Key: (●) epirubicin control; (○) epirubicin encapsulated in DPPC liposomes; (▼) epirubicin pretreated with empty DPPC liposomes; (▽) epirubicin pretreated with free DPPC; (▲) epirubicin encapsulated in DPPE liposomes; (Δ) epirubicin pretreated with empty DPPE liposomes; and (♦) epirubicin pretreated with free DPPE.

by different segments of the rat small intestine. The epirubicin concentrations measured in sacs treated with epirubicin liposomes or pretreated with empty liposomes before addition of epirubicin were significantly higher than those in the control groups in both the jejunum and the ileum ($P < 0.001$, N = 3 animals in each group). This implies a decrease in epirubicin efflux and/or an increase in epirubicin absorption. The enhancing effect of DPPE liposomes was superior to that of DPPC liposomes in both the jejunum and the ileum ($P < 0.01$, N = 3 animals in each group). Free DPPC or DPPE pretreatment showed marginal enhancement in epirubicin absorption ($P > 0.5$, N = 3).

DISCUSSION

In this study, we demonstrated that liposomal encapsulation and empty liposome pretreatment markedly increased the intracellular accumulation and apical-to-basolateral absorption of epirubicin across Caco-2 cells. These two formulations also substantially enhanced mucosal-to-serosal absorption of epirubicin in rat jejunum and ileum. Significant enhancement of these two treatments was also observed in the reduction of basolateral-to-apical secretion of epirubicin across Caco-2 monolayers. The functional involvement of P-gp in epirubicin efflux was verified by the addition of verapamil. Verapamil is a calcium channel blocker and a typical substrate of P-gp. It has been shown to competitively inhibit anticancer drug binding to P-gp, stimulate P-gp-associated ATPase, and reduce drug efflux from cells [2, 5]. Verapamil, combined with liposome formulations, intensified the pharmacological inhibition of P-gp and thus enhanced the intracellular accumulation of epirubicin in Caco-2 cells.

The polarized localization of P-gp has led to the suggestion that its physiological role is as a secretory detoxifying system responsible for the efflux of lipophilic molecules [20, 21]. In the small intestine, P-gp is present at high levels in the villus tips of enterocytes, the primary site of absorption for orally administered drugs. It is thus conceivable that P-gp can act as a barrier to the intestinal absorption of drugs by inducing a net basolateral-to-apical flux of xenobiotics [15, 18, 22, 23]. Although the precise mechanism of transport remains unclear, evidence suggests that this removal of drugs from the cytoplasm and/or membrane compartments may be due to physical translocation [5], electrochemical gradients [24], or flippase activity [25]. Furthermore, many P-gp substrates are hydrophobic and positively charged at neutral pH, and bind readily to negatively charged phospholipid head groups of the membrane [26]. Substrates appear to interact at overlapping sites of the putative transmembrane region 12 [27] and stimulate or inhibit P-gp ATPase activity [28].

Phospholipids are the major lipids in the plasma membranes of mammalian cells. Evidence shows that certain phospholipids play a critical role in generating second messengers for cell membrane signal transduction [29]. Disruptions in phospholipid metabolism can interfere with this process and may underlie certain disease states such as cancer and Alzheimer's disease [29]. Phospholipids have been proven to be the substrates of Class I and/or Class III P-gp in many cell lines [6–9]. In addition, human MDR1 P-gp has been shown to be a phospholipid translocase with broad specificity [7, 8, 30–32].

Thus, in this study, we suggest that both epirubicin and phospholipid molecules may be recognized by P-gp within the context of the lipid bilayer and may be translocated to the outer leaflet of the membrane or to the cell exterior, ultimately reducing their intracellular accumulation. Modulation of P-gp by phospholipids through substrate substitution, membrane fluidization, or other mechanisms may antagonize MDR and increase the cytotoxicity of epirubicin.

Drug transport by P-gp is energized by ATP hydrolysis at two highly conserved nucleotide-binding domains, which are located at the cytosolic face of the protein [33, 34]. Various phospholipids may change the lipid membrane environment, affect the ATPase activity of P-gp, and modulate the function of P-gp [33–37].

MDR-type drugs and modulators enter cells by passive diffusion through the plasma membrane. The resistance conferred upon MDR cells is the net result of the passive absorptive rate subtracted from the active secretory rate mediated by efflux transporter proteins, such as P-gp. This suggests that the balance between the influx and efflux mechanisms is an important factor in determining intestinal absorption [15]. The efficiency of P-gp in removing drugs from the MDR cells depends on the pumping capacity of P-gp and its affinity toward the specific drug, the transmembrane movement rate of the drug, the affinity of the drug toward its pharmacological cellular target, and intracellular trapping sites [38, 39]. Excellent modulators to overcome cellular P-gp may require the ability to be passively transported across the membrane rapidly and to re-enter the cell immediately, thus successfully occupying the P-gp active site(s) [38].

In our study, absorption of epirubicin was more limited when drug efflux exceeded diffusional influx. This was consistent with our experimental conditions before the addition of liposomes, when epirubicin had a low intrinsic passive permeability and a high affinity for ATP-dependent export. However, after treatment with liposomes, P-gp had a minimal limiting effect on epirubicin absorption when diffusional epirubicin influx was high with respect to epirubicin efflux. Drug influx was increased due to the higher intrinsic passive permeability of epirubicin after encapsulation into or pretreatment with liposomes. Drug efflux was reduced when the pump was saturated with substrates such as phospholipids, or when the function of the pump was inhibited by phospholipids.

Phospholipids have been suggested previously to be P-gp substrates [4, 6–10]. The binding affinities of drugs (or chemosensitizers) to P-gp are dependent on their ability to compete with the phospholipid substrates at the drug binding sites within the P-gp molecule [34]. Lipids (e.g. PC)

that interact weakly with P-gp may be more easily displaced, giving higher affinities for binding of drugs. However, tight-binding lipids (e.g. PE) may be more difficult to displace, resulting in lower drug-binding affinity [34]. This implies that use of high-affinity chemosensitizers to promote their interaction with P-gp in the tumor cells may antagonize MDR. This is consistent with our experimental conditions for improving enhancement of epirubicin absorption and increased reduction of epirubicin efflux by DPPE.

The passive transbilayer movement of most lipids, with the exception of diacylglycerol and cholesterol, is very slow [40]. The following three major plasma membrane proteins have been proposed to act as phospholipid translocators: aminophospholipid translocase, flippase, and lipid scramblase [40, 41]. Aminophospholipid translocase selectively transports PS and PE from the outer to the inner leaflet of the plasma membrane, but this transporter does not recognize PC. Flippase, e.g. MDR1 and/or MDR3 P-gp, is an outward-directed pump with little selectivity for the polar head group of the phospholipids. Lipid scramblase facilitates bi-directional migration of all phospholipid classes across the bilayer.

Liposomes may interact with cells using several mechanisms, including intermembrane transfer, adsorption, fusion, and endocytosis [42]. Epirubicin encapsulated in DPPC or DPPE liposomes may enter the cell surface through these mechanisms and thus markedly increase its absorption. Pretreatment with empty DPPC or DPPE liposomes allows PC or PE to be internalized into cells and to localize into the plasma membrane with a cell-specific asymmetric distribution between the two leaflets [43]. These liposomes may affect the cell association of drugs, change the transmembrane flux, or alter the binding of drugs to intracellular structures [44]. They may also change the fluidity of cell membranes and thus enhance the absorption of epirubicin.

Epirubicin encapsulated in or pretreated with DPPC or DPPE liposomes exhibited a substantial reduction in efflux across Caco-2 monolayers. These liposomal formulations may circumvent drug efflux by P-gp. However, exogenous free lipids with a long acyl chain length, e.g. C_{16} in our case, could not translocate across the plasma membranes. Free diffusion of these lipids across the membrane is very slow [40], which explains their marginal effects on both the absorptive and secretory directions of epirubicin transport. Conversely, lipid precursors, such as PA, or liposomal formulations could be used to enter the cells more easily [7, 8]. Due to the absence of a polar headgroup, PA molecules could translocate across the plasma membrane and, by monomeric exchange, reach the choline- and ethanolamine-phosphotransferase, probably at the endoplasmic reticulum, where PA is converted to PC or PE [45].

Our previous studies have shown that BSA works as an acceptor protein to extract phospholipids, including PC and PE, from the cell surface into the medium. This results in fewer P-gp substrates and more P-gp available for pumping epirubicin out of Caco-2 cells, thus decreasing the intracellular accumulation of epirubicin. BSA reverses the effects of MDR modulators, e.g. reserpine, on epirubicin efflux in Caco-2 cells. However, these effects were reversed by the addition of exogenous lipid precursors, e.g. PA and/or choline or ethanolamine [10, *].

In the current study, liposomal formulations, including liposomal encapsulation and empty liposome pretreatments, may enter the cell surface through endocytosis or other mechanisms to function as substrates of MDR1 and/or MDR3 P-gp, leaving less P-gp available for pumping epirubicin out of cells. These liposomal formulations may also induce a change in the composition and fluidity of cell membranes, and thus modulate the activity of membrane-spanning proteins such as P-gp.

Anticancer drugs encapsulated in liposomes have been found to provide tumor-specific delivery, as well as to circumvent P-gp-mediated drug resistance. They reduce toxicities by altering the pharmacodistribution properties of encapsulated drugs [46]. The improved anti-cancer activity is due, in part, to the increase of intracellular concentrations of the cytotoxic agents by the inhibition of P-gp-mediated drug efflux [1]. In addition, liposomes composed of phospholipids inhibit [^{3}H]vinblastine binding to plasma membranes and increase the intracellular accumulation of [^{3}H]vinblastine in MDR human lymphoblastic leukemic cells [47].

Some other drug efflux pump proteins, such as MDR-associated protein (MRP1), lung resistance protein (LRP), and the canalicular multispecific organic anion transporter (cMOAT), are found in the intestines [48, 49]. These transporter proteins are also present in Caco-2 cells [50, 51]. Their distribution overlaps significantly with that of P-gp. The overexpression of these energy-dependent pump proteins is thought to be involved in the development of MDR [50–52]. Recently, MRP1 has been shown to function as a lipid flippase to translocate phospholipids outside the erythrocyte membrane [53, 54]. Evidence suggests that both P-gp and MRP confer resistance to a similar but not identical spectrum of natural product chemotherapeutic agents, including anthracyclines such as epirubicin [55].

Although we have demonstrated in this study the involvement of P-gp in epirubicin transport by the use of verapamil, there is a possibility that liposomes may influence MRP or other transporter proteins and thus change the uptake and efflux of epirubicin. Phospholipids may change the lipid composition and fluidity of cell membranes, resulting in conformational and functional changes in drug efflux pump proteins, such as MRP1, LRP, and cMOAT. Further studies need to be performed to verify the role of these transporter proteins in epirubicin transport.

In conclusion, epirubicin encapsulated in or pretreated with DPPC or DPPE liposomes not only increased intracellular accumulation and apical-to-basolateral absorption of epirubicin, but also reduced basolateral-to-apical secre-

* Lo YL, Hsu CY and Huang JD, Manuscript in preparation.

tion of epirubicin across Caco-2 monolayers. Enhancement also was observed with both liposome encapsulation and empty liposome pretreatment in mucosal-to-serosal absorption of epirubicin in rat jejunum and ileum. Conversely, because diffusion of free DPPC or DPPE lipids across the cell membrane is very slow, these free lipids showed marginal effects on absorption and/or secretion of epirubicin in both Caco-2 cells and rat gut sacs.

On the basis of overlapping physicochemical characteristics between epirubicin and phospholipids such as PC and PE, we suggest that P-gp transporters may recognize common structural determinants on their specific substrates and transport them by a similar mechanism, possibly related to the phospholipid translocase activity of P-gp. With this study, we suggest that modulation of P-gp by exogenous phospholipids in the form of liposomes antagonizes MDR through substrate competition, membrane perturbation, or other mechanisms and enhances cytotoxicity of epirubicin. Therapeutic use of phospholipids as excipients in drug formulations may reduce systemic side-effects and improve oral bioavailability of drugs in the MDR spectrum. The combined use of epirubicin with phospholipids has important possible applications to circumvent drug resistance in cancer chemotherapy. Phospholipids may function as MDR-reversing agents for the inhibition of intestinal P-gp and may have potential therapeutic use.

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