



## Effects of Sodium Deoxycholate and Sodium Caprate on the Transport of Epirubicin in Human Intestinal Epithelial Caco-2 Cell Layers and Everted Gut Sacs of Rats

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**ABSTRACT.** The effects of sodium deoxycholate (Deo-Na), a bile salt, and sodium caprate (Cap-Na), a fatty acid, on the transport of epirubicin were investigated in both the human colon adenocarcinoma (Caco-2) cell line and the everted gut sacs of the rat jejunum and ileum. The possible use of these two potent absorption enhancers as multidrug resistance (MDR) reversing agents also was examined. Epirubicin uptake experiments using a flow cytometer showed that Deo-Na and Cap-Na significantly increased the accumulation of epirubicin in Caco-2 cells. These two enhancers significantly increased apical to basolateral absorption of epirubicin across Caco-2 monolayers and mucosal to serosal absorption of epirubicin in the rat jejunum and ileum. Moreover, the addition of Deo-Na or Cap-Na significantly reduced the basolateral to apical efflux of epirubicin across Caco-2 monolayers. The co-presence of verapamil, one typical P-glycoprotein (P-gp) substrate, and Deo-Na or Cap-Na demonstrated further reduction of epirubicin efflux. The study suggests that inhibition of P-gp or other transporter proteins located in the intestines may be involved, at least partially, in the reduction of epirubicin efflux. In conclusion, the therapeutic efficacy of epirubicin may be improved by the use of such low toxicity excipients as absorption enhancers and MDR modulators in formulations. *BIOCHEM PHARMACOL* 59;6:665–672, 2000. © 2000 Elsevier Science Inc.

**KEY WORDS.** epirubicin; sodium deoxycholate; sodium caprate; Caco-2; rat intestine; P-glycoprotein

The ability of malignant cells to develop simultaneous resistance to multiple chemotherapeutic agents, known as MDR<sup>§</sup>, appears to be a major obstacle in the successful treatment of clinical tumors. MDR is mediated by the increased expression of energy-dependent drug efflux pumps, such as P-gp, multidrug resistance-associated protein (MRP1), lung resistance protein (LRP), and the canalicular multispecific organic anion transporter (cMOAT) in cancer cells [1, 2]. These transport proteins with broad substrate specificity also are expressed in the intestines. By returning a portion of absorbed drugs to the intestinal lumen, they may serve as barriers to absorption of solutes that are substrates [3].

P-gp is known to pump out natural product-derived anticancer agents such as anthracyclines, vinca alkaloids, and epipodophyllotoxins [4]. Inhibition of P-gp function

using MDR reversing agents that act by substrate competition, ATP depletion, or membrane perturbation may antagonize multidrug resistance and increase intestinal absorption as well as cytotoxicity of anticancer drugs [5]. Some lipophilic agents such as verapamil and progesterone have been found to reverse the MDR phenotype *in vitro* by directly competing with the anticancer drug binding site(s) of P-gp. However, clinical application of these agents has not been very successful. At the doses required to reverse the MDR phenotype, severe *in vivo* side-effects have occurred [6]. Therefore, it is important to develop new categories of MDR reversing agents with low toxicity. At clinically achieved concentrations, some pharmacologically inert surfactants such as Tween 80 and Cremophor EL have proven effective in reversing the MDR phenotype in cultured cells [7]. These surfactants usually act by changing the fluidity of cell membranes and thus inhibit activity of membrane-spanning proteins, such as P-gp [7].

Many synthetic or natural compounds with different physicochemical properties reportedly have improved the intestinal absorption of hydrophilic and/or lipophilic drugs. Among these compounds, bile salts, fatty acids, and surfactants have been verified as potent absorption enhancers [8]. The mechanisms of action of most of these compounds are

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<sup>§</sup> Abbreviations: MDR, multidrug resistance; P-gp, P-glycoprotein; Deo-Na, sodium deoxycholate; Cap-Na, sodium caprate; Caco-2, human colon adenocarcinoma; TEER, transepithelial electrical resistance; and DMEM, Dulbecco's modified Eagle's medium.

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relatively nonspecific and seem to involve both the transcellular and the paracellular pathways [8, 9].

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 influx rate subtracted from the active efflux rate mediated by P-gp. This would suggest that the balance between the absorptive and secretory mechanisms is an important factor in determining intestinal absorption.

In the present study, two potent absorption enhancers, Deo-Na and Cap-Na, were chosen for their verified absorption-improving effect on different epithelial models [8–13]. Cap-Na, the sodium salt of the saturated decanoic acid (C10), constitutes 2–3% of the fatty acids in dairy products [10]. Deo-Na is a naturally occurring anionic bile salt. The absorption-enhancing properties of Deo-Na and Cap-Na have been studied extensively [8–13]. Studies have suggested that Cap-Na and Deo-Na enhance the transepithelial transport of hydrophilic model compounds via the paracellular route and that of hydrophobic model compounds via both paracellular and transcellular routes [8, 12]. Recently, they have been used clinically as absorption enhancers in Japan, Denmark, and Sweden. There have been no reports of severe side-effects [8, 9, 13].

Epirubicin, an anthracycline anticancer drug, was selected as a model drug in this study. This compound, with inversion of the 4'-hydroxyl group on the sugar moiety, is the epimer of the anthracycline antibiotic doxorubicin. It has a more favorable therapeutic index than that of doxorubicin. There is less hematologic or cardiac toxicity at comparable doses [14, 15]. Because epirubicin is an amphiphilic compound, we speculated that Deo-Na and Cap-Na might improve the absorption of epirubicin via both hydrophilic paracellular and lipophilic transcellular routes.

Since epirubicin is known to be pumped out by P-gp, the possibilities of Deo-Na and/or Cap-Na acting as MDR reversing agents to increase the intracellular accumulation, transepithelial flux, and intestinal absorption of epirubicin were investigated in this study. The effects of Deo-Na and Cap-Na on the intracellular accumulation and transepithelial transport of epirubicin were investigated in intact Caco-2 cells. A flow cytometer was used to determine if the resistance could be modulated by Deo-Na and Cap-Na by measuring the change in the intracellular fluorescent epirubicin concentrations [16–18]. The Caco-2 cell line, which is derived from a human colorectal carcinoma, is a human intestinal epithelial cell line that differentiates spontaneously to enterocyte-like cells under conventional cell culture conditions [19]. Caco-2 cells have been found to contain three major permeability barriers to the absorption of drugs. The three permeability barriers are the 'unstirred' water layer, the junctional complex (including tight junctions) between the cells, and the cell membranes [20]. After a cultivation period of 3 weeks on permeable polycarbonate filters, polarized monolayers with apical brush borders and well-developed tight junctions were obtained [20]. In the current study, with Caco-2 monolay-

ers grown in Transwell filters as a model, the effects of Cap-Na and Deo-Na on the apical to basolateral or basolateral to apical transport of epirubicin were investigated. 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  $t = 0$  [21]. Furthermore, the functional involvement of P-gp in this process was verified by the addition of one conventional P-gp reversing agent, verapamil. In the presence of verapamil, a typical P-gp substrate, the effects of Deo-Na or Cap-Na on the transport of epirubicin were evaluated in both the absorptive and secretory directions. In addition, the effects of Cap-Na and Deo-Na on the intestinal absorption of epirubicin were investigated in everted gut sacs prepared from the rat jejunum and ileum.

## MATERIALS AND METHODS

### *Chemicals and Animals*

Deo-Na (minimum 97%) and Cap-Na (99–100%) were purchased from the Sigma Chemical Co. Epirubicin was purchased from Pharmacia & Upjohn. 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  $\text{NaHCO}_3$  (12 mM), 6 mL of 10% KCl (2.7 mM), 7.8 mL of 10%  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$  (1.1 mM), 3.9 mL of 5%  $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$  (0.42 mM), and 5.4 mL of 1 M  $\text{CaCl}_2$  (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%  $\text{CO}_2$  and 90% relative humidity.

### *Flow Cytometric Studies*

Measurements of intracellular epirubicin fluorescence were made as described before [16–18]. Cells ( $3 \times 10^5/\text{cm}^2$ ) were seeded into 24-well plates and incubated for 21 days. Cells were rinsed twice with PBS, pretreated with 1.2 mM Deo-Na or 10 mM Cap-Na, and incubated for 30 min at 37°. The experiment was initiated by adding 1  $\mu\text{g}/\text{mL}$  of epirubicin to the culture medium. After a 3-hr incubation at 37°, the cells were rinsed twice with ice-cold PBS. To obtain a single-cell suspension, Caco-2 cell monolayers were rinsed with 0.25% trypsin in a 1-mM EDTA solution. The cells were collected into centrifuge tubes and centri-

fused for 10 min at 1000 g. Then the cells were 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.** Caco-2 cells were seeded into 0.45- $\mu$ m-pore Transwell inserts at 80,000 cells/cm<sup>2</sup> and grown for 21 days. Cell monolayers were used when the TEER exceeded 300  $\Omega \cdot$ cm<sup>2</sup> [22, 23]. The TEER values of the Caco-2 cell monolayers were measured periodically using the MILLICELL electrical resistance system (Millipore Corp.) to monitor cell layer confluence and integrity of tight junctions. Cell integrity was also checked by measuring the transepithelial fluxes of the paracellular marker [<sup>14</sup>C]mannitol before, during, and after the experiment. The transport studies then were performed as modified from previous studies [24–26]. Briefly, the cells were rinsed twice with PBS and allowed to incubate at 37° in 0.4 mL of serum-free DMEM containing 1.2 mM Deo-Na, Deo-Na plus 20  $\mu$ M verapamil, 10 mM Cap-Na, or Cap-Na plus verapamil in the apical (donor) compartments for 30 min. Serum-free DMEM (1.5 mL) was added to the basolateral (receiver) compartments. To initiate the experiment, the donor solution was added with serum-free DMEM containing 100  $\mu$ g/mL of epirubicin, followed by incubation in the dark at 37°. Samples (0.2 mL) of 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.** Deo-Na, Deo-Na plus verapamil, Cap-Na, or Cap-Na plus verapamil was added to the basolateral compartments of the Transwell in a volume of 1.5 mL of serum-free DMEM for 30 min, as previously described [24–26]. The basolateral (donor) solution was added with 100  $\mu$ g/mL of epirubicin and mixed with gentle agitation. Serum-free DMEM (0.4 mL) was added to the apical compartments to initiate transport. Samples (0.2 mL) on 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 before [27, 28]. Male Sprague–Dawley (SD) rats weighing about 300 g were deprived of food for 1 day before the experiments, with double-distilled water freely available. 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, 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 either 12 mM Deo-Na or 100 mM Cap-Na. Then 100  $\mu$ g/mL of epirubicin was added at 37°. In each study, 200  $\mu$ L of the solution inside the sacs was taken every 10 min for 60 min. Each experiment was triplicated.

### Analysis of Concentration of Epirubicin by HPLC

The analytic method for epirubicin was modified from previous reports [29, 30]. Daunorubicin was used as an internal standard. The HPLC system consisted of a Hitachi L7100 pump equipped with a satellite 710B WISP automated injector (Millipore Co.), a 5- $\mu$ m Lichrospher column (25-cm length, 4-mm inside diameter, E. 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 daunomycin by peak height was compared with the calibration curve for quantitation.

## RESULTS

### Flow Cytometric Studies

Initial studies were carried out to assess the effects of Deo-Na and Cap-Na on the intracellular accumulation of epirubicin after 180 min of uptake in Caco-2 cells. Figure 1 demonstrates the effects of Deo-Na and Cap-Na on the cell number versus fluorescence intensity of epirubicin in Caco-2 cells. These two enhancers apparently shifted the fluorescence intensity level to the right of that seen for the epirubicin control. Deo-Na and Cap-Na markedly enhanced the uptake of epirubicin in Caco-2 cells. The enhancement factor of Deo-Na was  $1.81 \pm 0.09$ , and that of Cap-Na was  $1.62 \pm 0.08$ . The enhancement factor is the ratio of fluorescence intensity of epirubicin with Deo-Na or Cap-Na divided by fluorescence intensity of epirubicin control. Data are the means  $\pm$  SD of six independent experiments.

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

Figures 2A, 2B, 3A, and 3B show the transepithelial fluxes of 100  $\mu$ g/mL of epirubicin across Caco-2 cell monolayers

TABLE 1. Effects of 1.2 mM Deo-Na, 10 mM Cap-Na, 20  $\mu$ M verapamil, Deo-Na plus verapamil, or Cap-Na plus verapamil on apparent permeability coefficients ( $P_{app}$ ) of epirubicin in Caco-2 cells\*

Treatment	$P_{app}$ of epirubicin (cm/sec) $\times 10^{-6}$					
	$P_{app, a \rightarrow b}$	$\dagger$	$P_{app, b \rightarrow a}$	$\ddagger$	$P_{app, net}^{\ddagger}$	$P_{app, net}^{\ddagger}$
Control	$0.61 \pm 0.04$		$1.41 \pm 0.03$		$0.80 \pm 0.04$	$0.80 \pm 0.04$
Deo-Na	$1.09 \pm 0.05^{\S}$	$\uparrow 79\%$	$0.88 \pm 0.04^{\S}$	$\downarrow 38\%$	$-0.21 \pm 0.05^{\S}$	$0.06 \pm 0.05^{\S}$
Verapamil	$0.96 \pm 0.05^{\S}$	$\uparrow 57\%$	$0.83 \pm 0.04^{\S}$	$\downarrow 41\%$	$-0.13 \pm 0.05^{\S}$	$-0.13 \pm 0.05^{\S}$
Deo-Na + verapamil	$1.61 \pm 0.05^{\S}$	$\uparrow 164\%$	$0.69 \pm 0.04^{\S}$	$\downarrow 51\%$	$-0.92 \pm 0.05^{\S}$	$-0.47 \pm 0.05^{\S}$
Cap-Na	$0.86 \pm 0.05^{\S}$	$\uparrow 40\%$	$0.92 \pm 0.04^{\S}$	$\downarrow 35\%$	$0.06 \pm 0.05^{\S}$	$0.06 \pm 0.05^{\S}$
Verapamil + verapamil	$0.96 \pm 0.05^{\S}$	$\uparrow 57\%$	$0.83 \pm 0.04^{\S}$	$\downarrow 41\%$	$-0.13 \pm 0.05^{\S}$	$-0.13 \pm 0.05^{\S}$
Cap-Na + verapamil	$1.25 \pm 0.05^{\S}$	$\uparrow 104\%$	$0.78 \pm 0.04^{\S}$	$\downarrow 45\%$	$-0.47 \pm 0.05^{\S}$	$-0.47 \pm 0.05^{\S}$

\* Data represent the means  $\pm$  SD of 4 independent experiments.

$\dagger$  Percent increase or decrease was calculated as  $[(\tau_2 - \tau_1)/\tau_1] \times 100$ , where  $\tau_1$  is  $P_{app}$  of the epirubicin control and  $\tau_2$  is  $P_{app}$  of epirubicin after application of modulators.

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

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

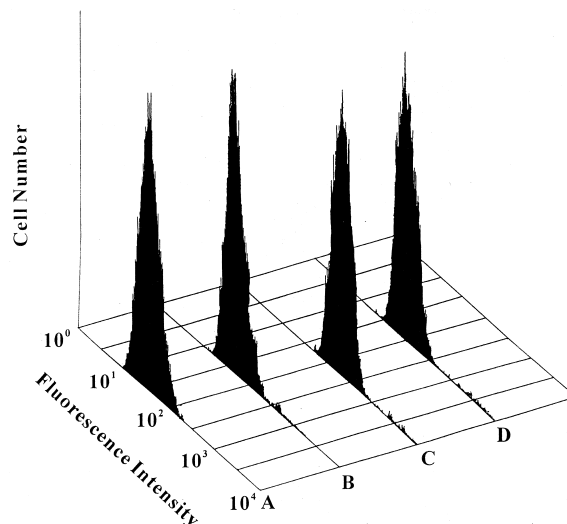


FIG. 1. Three-dimensional view of cell number versus fluorescence intensity of epirubicin in Caco-2 cells. Cells were pretreated with 1.2 mM Deo-Na or 10 mM Cap-Na for 30 min and incubated with 1  $\mu$ g/mL of epirubicin for 180 min. (A) cell control; (B) epirubicin control; (C) epirubicin pretreated with Deo-Na; and (D) epirubicin pretreated with Cap-Na.

at 37° in the absorptive (apical to basolateral;  $a \rightarrow b$ ) and secretory (basolateral to apical;  $b \rightarrow a$ ) directions plotted against time of incubation in the presence or absence of Deo-Na, Cap-Na, Deo-Na plus verapamil, or Cap-Na plus verapamil. The flux of epirubicin in the basolateral-to-apical direction was 2.29-fold of the flux in the apical-to-basolateral direction. A net flux of epirubicin, therefore, was observed in the secretory direction in Caco-2 cells. The demonstration of polarized transport correlated with the characteristics of P-gp substrates. This directional difference in fluxes was not observed with extracellular markers such as mannitol, for which transepithelial fluxes were approximately equal (data not shown), consistent with passive transepithelial mannitol flux. As demonstrated in Table 1, the apparent permeability coefficient ( $P_{app}$ ) of 100  $\mu$ g/mL of epirubicin in the absorptive direction was an order of magnitude less than that observed in the secretory direction. The net transport of epirubicin was thus in the secretory direction. The addition of Deo-Na shifted the net transepithelial transport of epirubicin from the secretory direction to the absorptive direction by both an increase in  $a \rightarrow b$   $P_{app}$  ( $\uparrow 79\%$ ) and a reduction of  $b \rightarrow a$   $P_{app}$  ( $\downarrow 38\%$ ). Therefore, Deo-Na showed a much more pronounced effect on enhancing the epirubicin absorption than on reducing the epirubicin efflux. The addition of Deo-Na in the presence of verapamil further shifted the epirubicin transport in the absorptive direction. The net  $P_{app}$  was given by both an increase in  $a \rightarrow b$   $P_{app}$  ( $\uparrow 164\%$ ) and a reduction of  $b \rightarrow a$   $P_{app}$  ( $\downarrow 51\%$ ).

As shown in Table 1, the addition of Cap-Na reduced the net secretory transport of epirubicin across the Caco-2 cells; net  $P_{app}$  was reduced to  $0.06 \times 10^{-6}$  cm/sec by both an increase in  $a \rightarrow b$   $P_{app}$  ( $\uparrow 40\%$ ) and a reduction of  $b \rightarrow a$



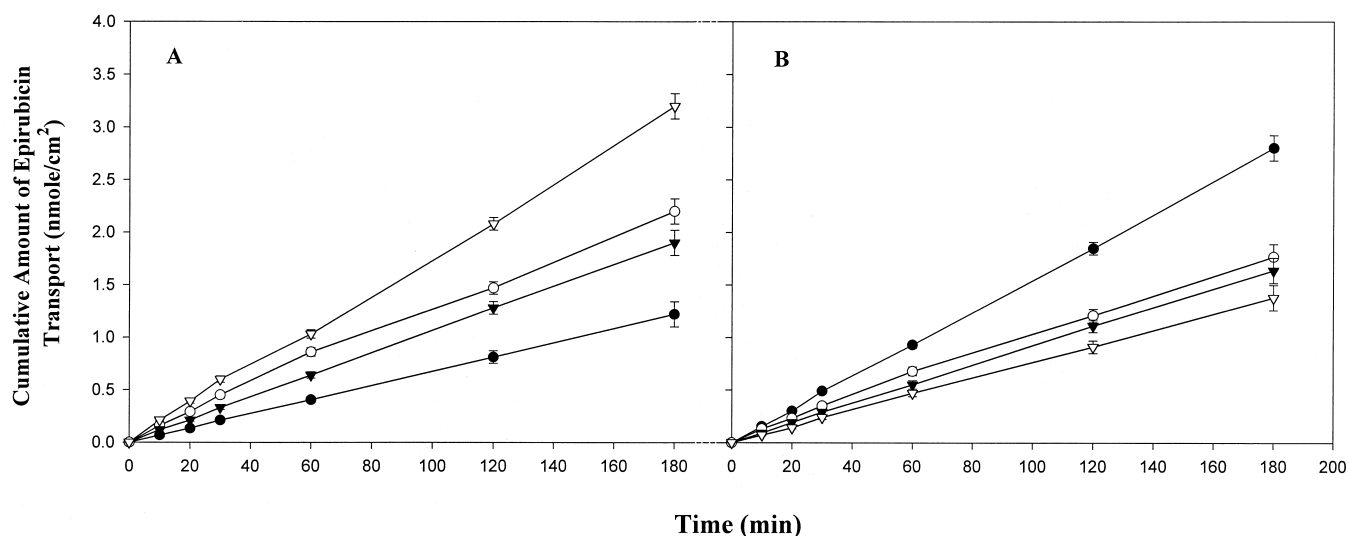


FIG. 2. Transepithelial fluxes of epirubicin across Caco-2 cell monolayers at 37° in the (A) absorptive (apical to basolateral; a→b) and (B) secretory (basolateral to apical; b→a) directions, plotted against time of incubation, in the presence or absence of Deo-Na and/or verapamil. Each point represents the mean  $\pm$  SD of four determinations. Key: (●) epirubicin control; (○) epirubicin pretreated with Deo-Na; (▼) epirubicin pretreated with verapamil; and (▽) epirubicin pretreated with Deo-Na plus verapamil.

$P_{app}$  ( $\downarrow$  35%). The addition of Cap-Na in the presence of verapamil shifted the epirubicin transport in the absorptive direction. The net  $P_{app}$  was obtained by both an increase in a→b  $P_{app}$  ( $\uparrow$  104%) and a reduction of b→a  $P_{app}$  ( $\downarrow$  45%).

#### Everted Sacs of Rat Jejunum and Ileum

As shown in panels A and B of Figure 4, epirubicin was transported from the mucosal side (bulk solution) to the serosal side (inside sac) in different segments of the small intestine. The epirubicin concentrations measured in sacs pretreated with Deo-Na or Cap-Na were significantly higher than those in the control groups in both the jejunum

and the ileum ( $P < 0.05$ ,  $N = 3$  animals in each group). This indicated a decrease in epirubicin efflux and/or an increase in epirubicin absorption. The enhancing effect of Deo-Na was superior to that of Cap-Na in both the jejunum and the ileum ( $P < 0.05$ ,  $N = 3$  animals in each group).

#### DISCUSSION

The results of our experiments indicated that Deo-Na and Cap-Na had enhancing effects on intracellular accumulation and apical to basolateral absorption across Caco-2 monolayers. These compounds also showed enhancement

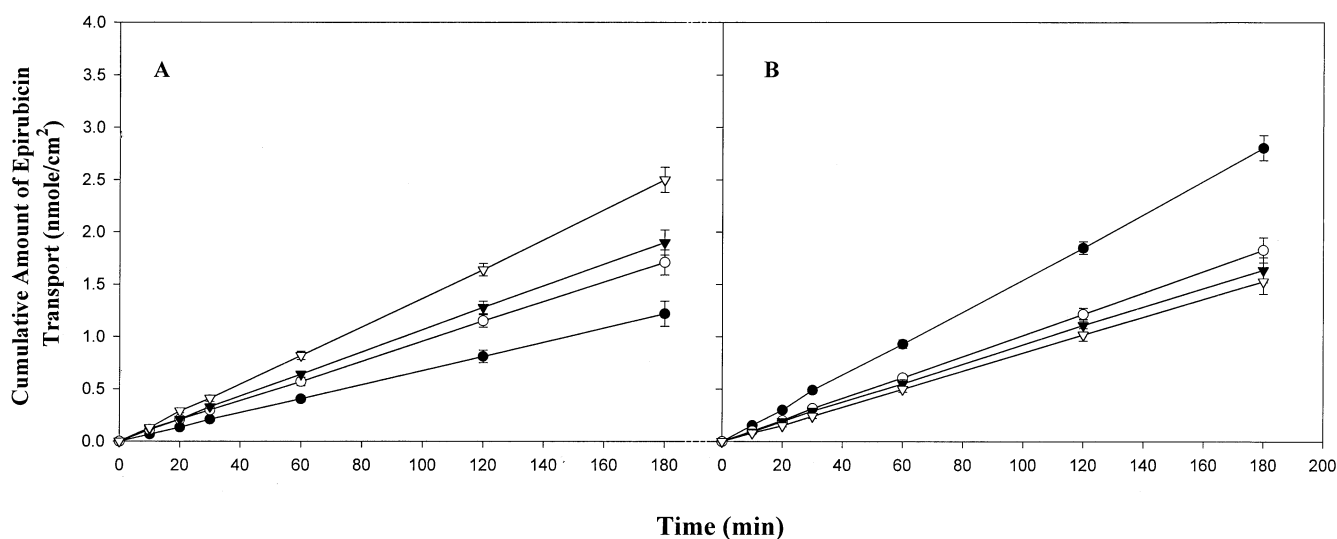
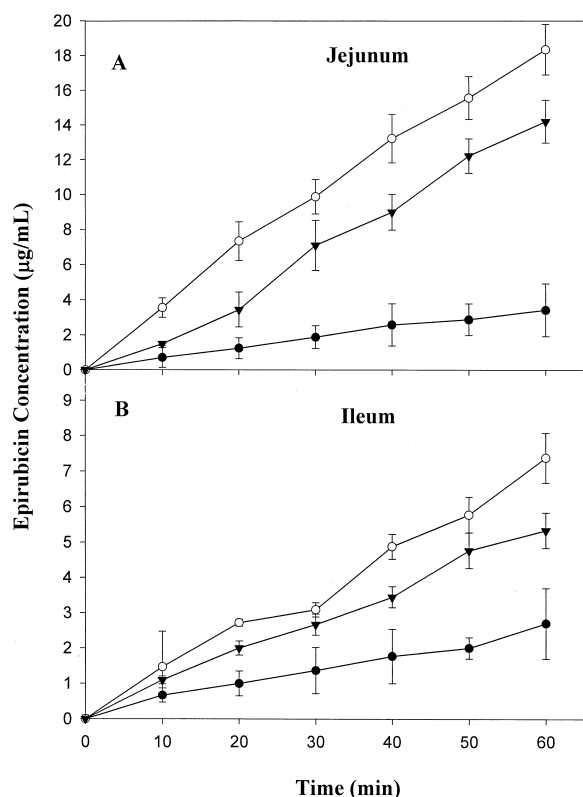


FIG. 3. Transepithelial fluxes of epirubicin across Caco-2 cell monolayers at 37° in the (A) absorptive (apical to basolateral; a→b) and (B) secretory (basolateral to apical; b→a) directions, plotted against time of incubation, in the presence or absence of Cap-Na and/or verapamil. Each point represents the mean  $\pm$  SD of four determinations. Key: (●) epirubicin control; (○) epirubicin pretreated with Cap-Na; (▼) epirubicin pretreated with verapamil; and (▽) epirubicin pretreated with Cap-Na plus verapamil.



**FIG. 4.** Time profile of epirubicin concentrations inside everted sacs of jejunum (A) and ileum (B) of rats in the presence or absence of modulators. Each point represents the mean  $\pm$  SD of triplicate determinations. Key: (●) epirubicin control; (○) epirubicin pretreated with Deo-Na; and (▼) epirubicin pretreated with Cap-Na.

of mucosal to serosal absorption of epirubicin in the rat jejunum and ileum.

A previous study has suggested that the transepithelial permeability of a P-gp substrate depends not only on the passive permeability of the apical membrane to the substrate, but also on its affinity for the active transport site and the maximal capacity of P-gp contained in the apical membrane [22]. With respect to 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 Deo-Na or Cap-Na, when epirubicin had a low intrinsic passive permeability and a high affinity for ATP-dependent export. However, after the addition of Deo-Na or Cap-Na, 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. Drug efflux was reduced when the pump was saturated with substrates or when the function of the pump was inhibited by modulators.

Deo-Na and Cap-Na have been verified as potent absorption enhancers that act by improving drug absorption via hydrophilic paracellular and/or lipophilic transcellular routes [31]. Cap-Na enhances paracellular drug absorption

by opening the tight junctions through an increase in the membrane pore radius, widening of the intercellular space [32], contraction of calmodulin-dependent actin microfilaments [33, 34], or contraction of the perijunctional actomyosin ring [12]. Fatty acids, such as Cap-Na, also have been reported to have metal-chelating ability. They may inhibit phospholipase C, inhibit or activate various kinases, and induce the release of  $\text{Ca}^{2+}$  from intracellular stores [9]. They may also make the intercellular space more accessible by temporarily eliminating calcium ions from the intestinal mucosa, resulting in partial destruction of tight junctions [35].

Deo-Na may enhance drug permeability by markedly reducing the length and distribution of glycocalyx filaments in the microvilli of rat intestinal epithelial cells [36]. A previous study reported that Deo-Na improves the nonselective paracellular transport of horseradish peroxidase through loosening of the tight junctions [37]. A confocal laser scanning study of Caco-2 cell monolayers revealed that Cap-Na and Deo-Na enhance the transepithelial transport of hydrophilic model compounds via the paracellular route and that of hydrophobic model compounds via both paracellular and transcellular routes. These two enhancers affect the transcellular route by creating membrane perturbation, which results from their interaction with membrane lipids and/or proteins. They also affect the paracellular route by opening tight junctions [8, 32, 38].

Our experiment is the first in the literature to demonstrate that Deo-Na and Cap-Na decreased the basolateral to apical secretion of epirubicin across Caco-2 monolayers. In the gut, P-gp is localized in the apical membrane of enterocytes. Its expression increases from crypt to villus, the primary site of absorption for orally administered drugs. Thus, it is conceivable that by inducing a net basolateral to apical flux of xenobiotics, P-gp can act as a barrier to the intestinal absorption of drugs [18, 26, 27, 39]. P-gp is known to be located in the apical (brush-border) membrane of Caco-2 cells. Epirubicin is one analog of the anthracycline antibiotic doxorubicin and is known to be pumped out by P-gp. We thus speculated that the function of P-gp and/or other active transport systems responsible for the efflux of epirubicin in the intestines might be influenced by the presence of Deo-Na or Cap-Na.

P-gp is a 170-kDa membrane glycoprotein, which includes two ATP binding domains. It has approximately twelve transmembrane segments [4]. Inhibition of P-gp function using MDR reversing agents that act by substrate competition, ATP-depletion, or membrane perturbation may antagonize multidrug resistance. Previous research has shown that Deo-Na and Cap-Na may produce membrane perturbation in rat colon epithelium and Caco-2 cells [8, 38]. Our study suggests that such membrane perturbation caused by Deo-Na and Cap-Na may result in a change in the fluidity of Caco-2 cell membranes, and thus inhibit the activity of membrane-spanning proteins, such as P-gp. Other MDR reversing agents, such as surfactants, have a similar effect [7]. In addition, Cap-Na has been shown to reduce cellular dehydrogenase activity and ATP levels [9].

This might result in energy depletion for ATP-dependent proteins such as P-gp, and thus affect its ability to act as a drug efflux pump. This effect was consistent with our observations that Deo-Na and Cap-Na decreased the basolateral to apical secretion of epirubicin across Caco-2 monolayers. Furthermore, the functional involvement of P-gp in this process was verified by the addition of verapamil. Verapamil, a calcium channel blocker, is a typical P-gp substrate. It is one of the most extensively studied MDR modulators [6]. Studies have suggested that verapamil competes with other substrates for binding to P-gp and stimulates P-gp-associated ATPase [4, 5]. The co-presence of verapamil and Deo-Na/or Cap-Na intensified the pharmacological inhibition of P-gp and thus demonstrated the further reduction in epirubicin efflux.

The reduction in the TEER of Caco-2 monolayers and the increase in the permeability coefficients of 1  $\mu$ Ci/mL of [ $^{14}$ C]mannitol across Caco-2 monolayers during the experiment (data not shown) suggest that Deo-Na and Cap-Na may affect the paracellular route through the opening of tight junctions and reduce the cell integrity of Caco-2 cells. However, the recovery of the TEER and the  $P_{app}$  of [ $^{14}$ C]mannitol after removal of these two modulators (data not shown) was consistent with their reversible effect on tight junction widening. This effect has been observed for substances such as EDTA and sodium caprate in previous studies [40, 41]. Deo-Na and Cap-Na appeared to enhance transepithelial transport of epirubicin, an amphiphilic compound, via both paracellular and transcellular routes. These two modulators significantly increased the apical to basolateral absorption of epirubicin and markedly reduced the basolateral to apical efflux of epirubicin. The study suggests that inhibition of P-gp or other transporter proteins located in the intestines may have at least a partial role in the reduction of epirubicin efflux in the secretory direction.

Further studies need to be performed to confirm the mechanisms involved in the MDR modulating phenomenon mediated by these two modulators and to evaluate the clinical benefit of the combined use of epirubicin with these modulators.

In conclusion, Deo-Na and Cap-Na not only enhanced transcellular and paracellular absorption of epirubicin, but also reduced the basolateral to apical secretion of epirubicin. Their enhancing properties were shown in intracellular accumulation and transport of epirubicin across Caco-2 monolayers. Enhancement was also observed in mucosal to serosal absorption of epirubicin in rat jejunum and ileum. This study suggests that Deo-Na and Cap-Na may have MDR reversing effects. Therapeutic use of these compounds as excipients for the inhibition of intestinal P-gp may improve the oral bioavailability of epirubicin. As MDR-reversing agents in drug formulations, they may reduce systemic side-effects and improve oral absorption of drugs. More importantly, the combined use of chemotherapeutic agents with Deo-Na or Cap-Na may have significant implications in circumventing drug resistance in cancer chemotherapy.

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