行政院國家科學委員會補助專題研究計畫成果報告

※※※※※※※※※※※※※※※※※※※※※※※※※※

※ **藥學賦形劑對P醣蛋白藥物運送之影響及機轉探討** ※

※ **The effects and mechanisms of pharmaceutical excipients** ※

※ **on the drug transport by P-glycoprotein** ※

※※※※※※※※※※※※※※※※※※※※※※※※※※

計畫類別：☑個別型計畫　　□整合型計畫

計畫編號：NSC 89-2314-B-041-002

執行期間：88年8月1日至89年7月31日

計畫主持人：駱雨利副教授

共同主持人：

本成果報告包括以下應繳交之附件：

□赴國外出差或研習心得報告一份

□赴大陸地區出差或研習心得報告一份

□出席國際學術會議心得報告及發表之論文各一份

□國際合作研究計畫國外研究報告書一份

執行單位：嘉南藥理科技大學藥學系

中　華　民　國 89年10月23日

行政院國家科學委員會專題研究計畫成果報告
藥學賦形劑對 P 醣蛋白藥物運送之影響及機轉探討
The effects and mechanisms of pharmaceutical excipients on the drug transport by P-glycoprotein

計畫編號: NSC 89-2314-B-041-002
執行期限: 88 年 8 月 1 日至 89 年 7 月 31 日
主持人: 駱雨利副教授　　嘉南藥理科技大學藥學系

一、中文摘要

本研究之主要目的是評估膽鹽 sodium deoxycholate (Deo-Na) 和脂肪酸 sodium caprate (Cap-Na)對 epirubicin 於人類結腸腺癌細胞(Caco-2)及老鼠小腸運輸之影響，並探討開發此二種吸收促進劑以作為多重抗藥性拮抗劑之可行性。使用流式細胞分析儀，顯示 Deo-Na 和 Cap-Na 明顯增進 epirubicin 於 Caco-2 細胞之積聚。以 Caco-2 細胞為小腸運輸模型，發現 Deo-Na 和 Cap-Na 可顯著促進 epirubicin 於吸收方向之運輸及明顯減少 epirubicin 於排出方向之運輸。同時加入傳統 P 醣蛋白受質 verapamil 和 Deo-Na 或 Cap-Na，則進一步促進 epirubicin 之吸收及減少 epirubicin 之排出。老鼠小腸實驗證實，不管是在空腸或迴腸，Deo-Na 和 Cap-Na 均能明顯增進 epirubicin 之吸收。本篇研究顯示抑制小腸 P 醣蛋白或其他排出藥物之蛋白質可能跟 epirubicin 之增加吸收及減少排出有關。總結，臨床應用 Deo-Na 和 Cap-Na 以當作吸收促進劑及多重抗藥性抑制劑可以促進 epirubicin 之小腸吸收，並可應用於拮抗癌症化學療法上之多重抗藥性。使用這些低毒性之賦形劑於劑型中可增進 P 醣蛋白受質之生體可用率並具有無全身性副作用及因改善藥物口服吸收所帶來之服用方便的優點。

關鍵詞： Epirubicin、Sodium deoxycholate、Sodium caprate、結腸腺癌細胞、老鼠小腸、P 醣蛋白、多重抗藥性

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 increase 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.

Keywords: epirubicin; sodium deoxycholate; sodium caprate; Caco-2; everted gut sacs; P-glycoprotein; multidrug resistance

二、緣由及目的

The ability of malignant cells to develop simultaneous resistance to multiple chemotherapeutic agents, known as multidrug resistance (MDR), 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[(1)].

Sodium caprate (Cap-Na), the sodium salt of the saturated decanoic acid (C10), constitutes 2-3 % of the fatty acids in dairy products. Sodium deoxycholate (Deo-Na) is a naturally occurring anionic bile salt. Recently, they have been used clinically as absorption enhancers in Japan, Denmark, and Sweden. There have been no reports of severe side-effects[(2)].

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 Caco-2 cells as well as everted gut sacs of rats. Furthermore, the functional involvement of P-gp in this process was verified by the addition of one conventional P-gp reversing agent, verapamil.

三、結果及討論

As demonstrated in Figs. 1 and 2, the addition of Deo-Na or Cap-Na significantly increased the intracellular accumulation and mucosal to serosal absorption of epirubicin in Caco-2 cells and everted gut sacs of rats.

Fig. 3 and Table 1 show that the addition of Deo-Na or Cap-Na apparently enhanced apical to basolateral (a→b) absorption and significantly reduced basolateral to apical (b→a) efflux of epirubicin across Caco-2 monolayers. The co-presence of verapamil, one typical P-gp substrate, and Deo-Na or Cap-Na demonstrated further reduction of epirubicin efflux. In all these studies, the enhancing effect of Deo-Na was superior to that of Cap-Na.

Deo-Na and Cap-Na have been verified as potent absorption enhancers. Previous studies revealed that Cap-Na and Deo-Na enhanced 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 affect the paracellular route by opening tight junctions [(3)].

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. P-gp is known to be located in the apical (brush-border) membrane of Caco-2 cells[(4)]. 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.

Previous research has shown that Deo-Na and Cap-Na may produce membrane perturbation in rat colon epithelium and Caco-2 cells[5]. 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. In addition, Cap-Na has been shown to reduce cellular dehydrogenase activity and ATP levels[2]. 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[6]. Furthermore, the functional involvement of P-gp in epirubicin efflux was verified by the addition of verapamil. Verapamil, a calcium channel blocker, is a typical P-gp substrate[7]. 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.

Our study implies 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.

In conclusion, our study suggests that Deo-Na and Cap-Na may have MDR reversing effects. Therapeutic use of these 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. The combined use of chemotherapeutic agents with Deo-Na or Cap-Na may have significant implications in circumventing drug resistance in cancer chemotherapy.

四、計畫成果自評

在應用價值方面，本計畫佐以細胞及動物試驗來評估賦形劑對抗癌藥物吸收及排出之影響，此部分實驗將提供臨床合併使用抗癌藥物及賦形劑為多重抗藥性抑制劑作治療之依據。

在學術價值方面，在這個研究計畫中，藉由不同賦形劑與 epirubicin 之交互作用之研究，我們已建立適當之老鼠小腸及人體小腸細胞吸收之模型並期望能推廣到其它抗癌藥物以發現更多低毒性之多重抗藥性抑制劑，進而提高癌症化學療法的成功率。

本篇成果報告僅節錄其中二個賦形劑與 epirubicin 作用的結果。其它賦形劑與 epirubicin 作用的結果也已完成。綜合其它賦形劑類多重抗藥性抑制劑之結果，將可得到全面性之結論。這些結果目前已發表一篇文獻於 Biochemical Pharmacology[8]上，其它尚在整理階段，將發表於學術期刊上，並亟具有臨床應用之遠景。

五、參考文獻

1. Germann UA. Eur J Cancer 32A: 927-944, 1996.
2. Lindmark T, Kimura Y and Artursson P. J Pharmacol Exp Ther 284: 362-369, 1998.
3. Sakai M, Imai T, Azuma H and Otagiri M. J Pharm Sci 86: 779-785, 1997.
4. Hunter J, Hirst BH and Simmons NL. Pharm Res 10: 743-749, 1993.
5. Sawada T, Ogawa T, Hayashi M and Awazu S. Pharm Res 8: 1365-1371, 1991.
6. Ford JM and Hait WN. Cytotechnology 12: 171-212, 1993.
7. Gottesman MM and Pastan I. Annu Rev Biochem 62: 386-427, 1993.
8. Lo YL and Huang JD. Biochem. Pharmacol. 59: 665-672, 2000.

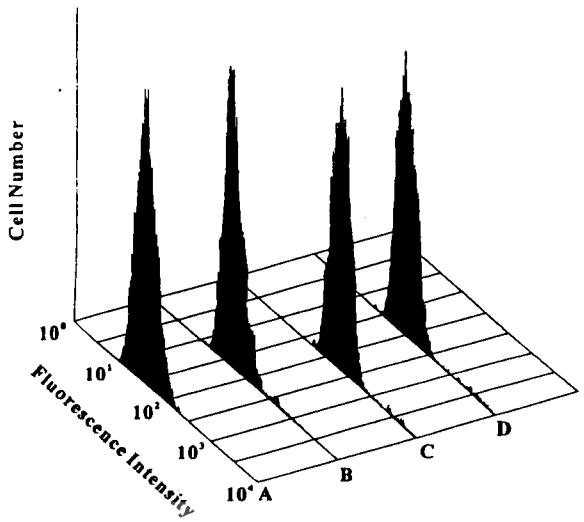

FIG. 1. Three-dimensional view of cell number versus fluorescence intensity of epirubicin in Caco-2 cells. Cells were pretreated with 1.2 mM Deo-Na or 10 mM Cap-Na for 30 min and incubated with 1 μg/mL of epirubicin for 180 min. (A) cell control; (B) epirubicin control; (C) epirubicin pretreated with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

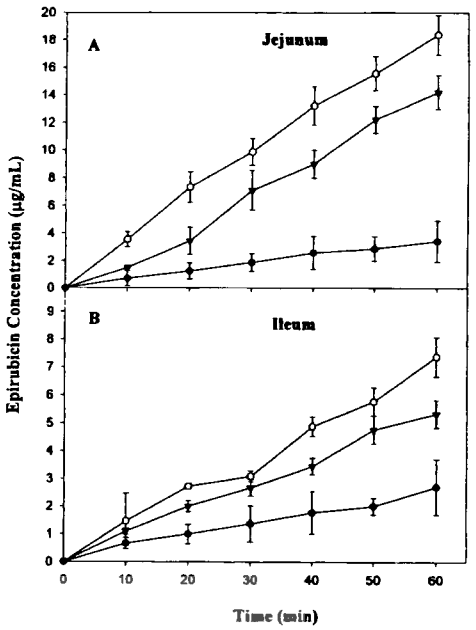

FIG. 2. Time profile of epirubicin concentrations inside everted sacs of jejunum (A) and ileum (B) of rats in the presence or absence of modulators. Each point represents the mean ± SD of triplicate determinations. Key: (●) epirubicin control; (○) epirubicin pretreated with Deo-Na; and (▼) epirubicin pretreated with Cap-Na.

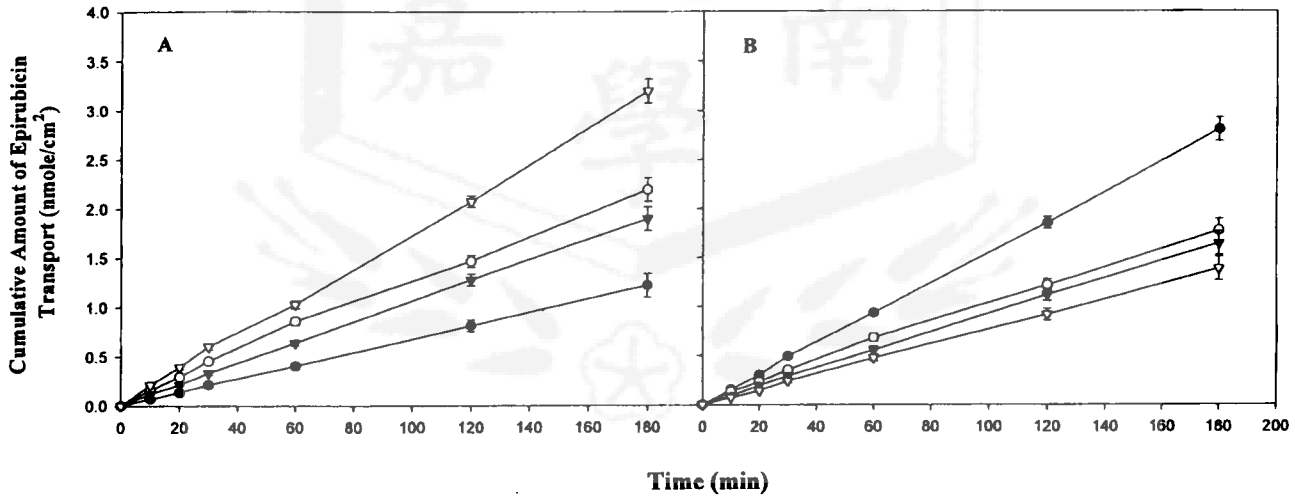

FIG. 3. Transepithelial fluxes of epirubicin across Caco-2 cell monolayers at 37° in the (A) absorptive (apical to basolateral; a→b) and (B) secretory (basolateral to apical; b→a) directions, plotted against time of incubation, in the presence or absence of Deo-Na and/or verapamil. Each point represents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

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

	P_{app} of epirubicin (cm/sec) × 10^{-6}										
Treatment	$P_{app, a\to b}$	†	$P_{app, b\to a}$	†	$P_{app, net}$‡	Treatment	$P_{app, a\to b}$	†	$P_{app, b\to a}$	†	$P_{app, net}$‡
Control	0.61 ± 0.04		1.41 ± 0.03		0.80 ± 0.04	Control	0.61 ± 0.04		1.41 ± 0.03		0.80 ± 0.04
Deo-Na	1.09 ± 0.05§	↑ 79%	0.88 ± 0.04§	↓ 38%	−0.21 ± 0.05§	Cap-Na	0.86 ± 0.05§	↑ 40%	0.92 ± 0.04§	↓ 35%	0.06 ± 0.05§
Verapamil	0.96 ± 0.05§	↑ 57%	0.83 ± 0.04§	↓ 41%	−0.13 ± 0.05§	Verapamil	0.96 ± 0.05§	↑ 57%	0.83 ± 0.04§	↓ 41%	−0.13 ± 0.05§
Deo-Na + verapamil	1.61 ± 0.05§	↑ 164%	0.69 ± 0.04§	↓ 51%	−0.92 ± 0.05§	Cap-Na + verapamil	1.25 ± 0.05§	↑ 104%	0.78 ± 0.04§	↓ 45%	−0.47 ± 0.05§

* Data represent the means ± SD of 4 independent experiments.
† Percent increase or decrease was calculated as $[(\tau_2 - \tau_1)/\tau_1] \times 100$, where τ_1 is P_{app} of the epirubicin control and τ_2 is P_{app} of epirubicin after application of modulators.
‡ $P_{app, net}$ values were calculated as $P_{app, net} = P_{app, b\to a} - P_{app, a\to b}$. Positive values represented net mucosal secretion, and negative values indicated net mucosal absorption of epirubicin.
§Significantly different from the control by Student's t-test ($P < 0.05$).

Biochemical Pharmacology, Vol. 59, pp. 665–672, 2000.

ISSN 0006-2952/00/$–see front matter
PII S0006-2952(99)00377-9

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

Yu-Li Lo† and Jin-Ding Huang‡*

*Department of Pharmacy, Chia-Nan College of Pharmacy and Science, Tainan Hsien 71710, Taiwan, R.O.C.; and ‡Department of Pharmacology, National Cheng Kung University Medical College, Tainan 70101, Taiwan, R.O.C.

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§, 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

† Corresponding author: Yu-Li Lo, Ph.D., Department of Pharmacy, Chia-Nan College of Pharmacy and Science, 60 Erh-Jen Road, Sec. 1, Jen-Te Hsiang, Tainan Hsien, Taiwan 71710. Tel. 011-886-6-2664911; FAX 011-886-6-2666411; E-mail: yulilo@ms17.hinet.net

§ *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.

Received 27 April 1999; accepted 11 August 1999.

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 monolayers 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 $NaHCO_3$ (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.

Flow Cytometric Studies

Measurements of intracellular epirubicin fluorescence were made as described before [16–18]. Cells ($3 \times 10^5/cm^2$) 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 μg/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-

fuged 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-μm-pore Transwell inserts at 80,000 cells/cm^2 and grown for 21 days. Cell monolayers were used when the TEER exceeded 300 Ω ·cm^2 [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 [^{14}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 μM 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 μg/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 μg/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 μg/mL of epirubicin was 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 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-μm 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 ± 0.09, and that of Cap-Na was 1.62 ± 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 ± 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 μg/mL of epirubicin across Caco-2 cell monolayers

TABLE 1. Effects of 1.2 mM Deo-Na, 10 mM Cap-Na, 20 μM 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) × 10^{-6}								
	$P_{app, a \to b}$	†	$P_{app, b \to a}$	†	$P_{app, net}$‡	Treatment	$P_{app, a \to b}$	†	$P_{app, b \to a}$	†	$P_{app, net}$‡
Control	0.61 ± 0.04		1.41 ± 0.03		0.80 ± 0.04	Control	0.61 ± 0.04		1.41 ± 0.03		0.80 ± 0.04
Deo-Na	1.09 ± 0.05§	↑ 79%	0.88 ± 0.04§	↓ 38%	−0.21 ± 0.05§	Cap-Na	0.86 ± 0.05§	↑ 40%	0.92 ± 0.04§	↓ 35%	0.06 ± 0.05§
Verapamil	0.96 ± 0.05§	↑ 57%	0.83 ± 0.04§	↓ 41%	−0.13 ± 0.05§	Verapamil	0.96 ± 0.05§	↑ 57%	0.83 ± 0.04§	↓ 41%	−0.13 ± 0.05§
Deo-Na + verapamil	1.61 ± 0.05§	↑ 164%	0.69 ± 0.04§	↓ 51%	−0.92 ± 0.05§	Cap-Na + verapamil	1.25 ± 0.05§	↑ 104%	0.78 ± 0.04§	↓ 45%	−0.47 ± 0.05§

* Data represent the means ± SD of 4 independent experiments.
† Percent increase or decrease was calculated as $[(\tau_2 - \tau_1)/\tau_1] \times 100$, where τ_1 is P_{app} of the epirubicin control and τ_2 is P_{app} of epirubicin after application of modulators.
‡ $P_{app, net}$ values were calculated as $P_{app, net} = P_{app, b \to a} - P_{app, a \to b}$. Positive values represented net mucosal secretion, and negative values indicated net mucosal absorption of epirubicin.
§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 μg/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→b) and secretory (basolateral to apical; b→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 μg/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→b P_{app} (↑ 79%) and a reduction of b→a P_{app} (↓ 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→b P_{app} (↑ 164%) and a reduction of b→a P_{app} (↓ 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 × 10^{-6} cm/sec by both an increase in a→b P_{app} (↑ 40%) and a reduction of b→a

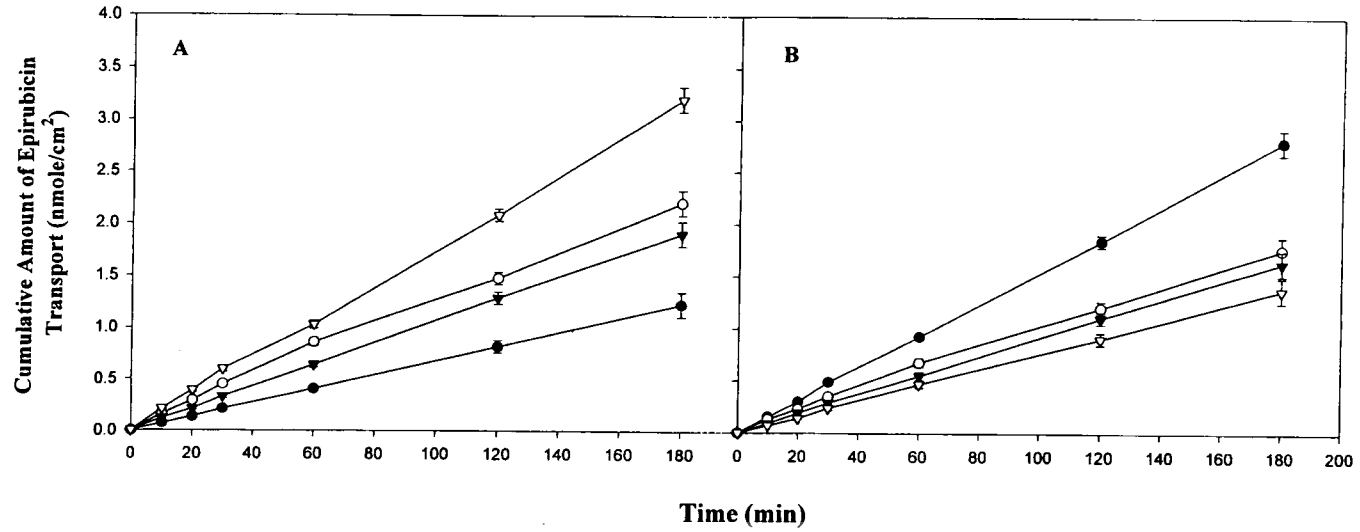

FIG. 2. Transepithelial fluxes of epirubicin across Caco-2 cell monolayers at 37° in the (A) absorptive (apical to basolateral; a→b) and (B) secretory (basolateral to apical; b→a) directions, plotted against time of incubation, in the presence or absence of Deo-Na and/or verapamil. Each point represents the mean ± 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} (↓ 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} (↑ 104%) and a reduction of b→a P_{app} (↓ 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

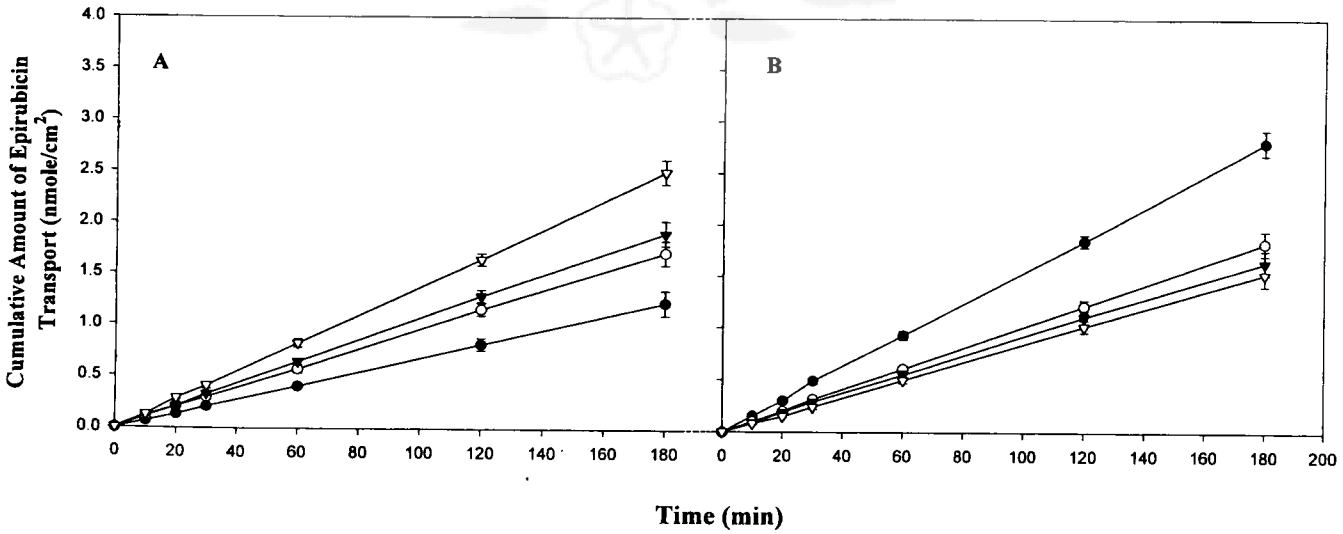

FIG. 3. Transepithelial fluxes of epirubicin across Caco-2 cell monolayers at 37° in the (A) absorptive (apical to basolateral; a→b) and (B) secretory (basolateral to apical; b→a) directions, plotted against time of incubation, in the presence or absence of Cap-Na and/or verapamil. Each point represents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

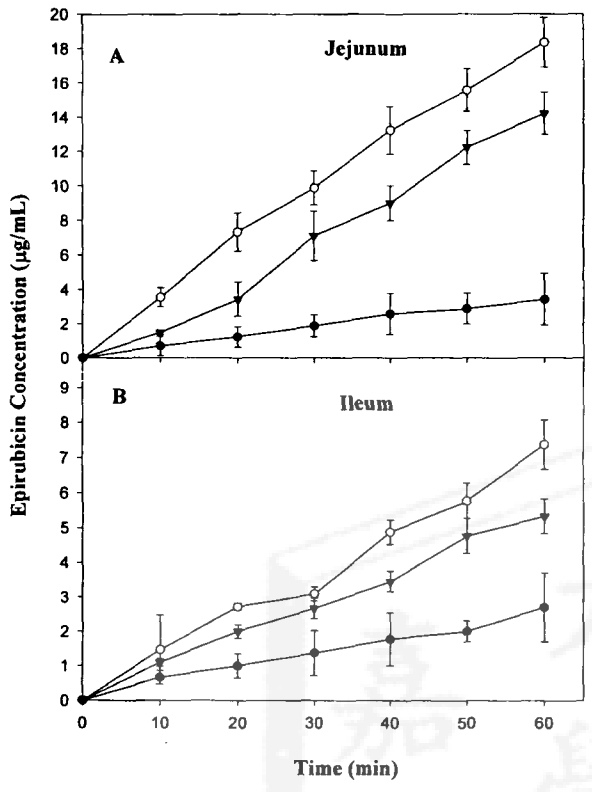

FIG. 4. Time profile of epirubicin concentrations inside everted sacs of jejunum (A) and ileum (B) of rats in the presence or absence of modulators. Each point represents the mean ± SD of triplicate determinations. Key: (●) epirubicin control; (○) epirubicin pretreated with Deo-Na; and (▼) epirubicin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

This work was supported by Grant NSC 89-2314-B-041-002 from the National Science Council, Republic of China.

References

1. Sagawara I, Akiyama S, Scheper RJ and Itoyama S, Lung resistance protein (LRP) expression in human normal tissues in comparison with that of MDR1 and MRP. *Cancer Lett* **112:** 23–31, 1997.
2. Ito K, Suzuki H, Hirohashi T, Kume K, Shimizu T and Sugiyama Y, Molecular cloning of canalicular multispecific organic anion transporter defective in EHBR. *Am J Physiol* **272:** G16–G22, 1997.
3. Makhey VD, Guo A, Norris DA, Hu P, Yan J and Sinko PJ, Characterization of the regional intestinal kinetics of drug efflux in rat and human intestine and in Caco-2 cells. *Pharm Res* **15:** 1160–1167, 1998.
4. Ford JM and Hait WN, Pharmacology of drugs that alter multidrug resistance in cancer. *Pharmacol Rev* **42:** 155–199, 1990.
5. Gottesman MM and Pastan I, Biochemistry of multidrug resistance mediated by the multidrug transporter. *Annu Rev Biochem* **62:** 386–427, 1993.
6. Germann UA, P-glycoprotein—A mediator of multidrug resistance in tumour cells. *Eur J Cancer* **32A:** 927–944, 1996.
7. Dudeja PK, Anderson KM, Harris JS, Buckingham L and Coon JS, Reversal of multidrug resistance phenotype by surfactants: Relationship to membrane lipid fluidity. *Arch Biochem Biophys* **319:** 309–315, 1995.
8. Sakai M, Imai T, Ohtake H, Azuma H and Otagiri M, Effects of absorption enhancers on the transport of model compounds in Caco-2 cell monolayers: Assessment by confocal laser scanning microscopy. *J Pharm Sci* **86:** 779–785, 1997.
9. Lindmark T, Kimura Y and Artursson P, Absorption enhancement through intracellular regulation of tight junction permeability by medium chain fatty acids in Caco-2 cells. *J Pharmacol Exp Ther* **284:** 362–369, 1998.
10. Soderholm JD, Oman H, Blomquist L, Veen J, Lindmark T and Olaison G, Reversible increase in tight junction permeability to macromolecules in rat ileal mucosa *in vitro* by sodium caprate, a constituent of milk fat. *Dig Dis Sci* **43:** 1547–1552, 1998.
11. Argenzio RA, Henrikson CK and Liacos JA, Effect of prostaglandin inhibitors on bile salt-induced mucosal damage of porcine colon. *Gastroenterology* **96:** 95–109, 1989.
12. Shimazaki T, Tomita M, Sadahiro S, Hayashi M and Awazu S, Absorption-enhancing effects of sodium caprate and palmitoyl carnitine in rat and human colons. *Dig Dis Sci* **43:** 641–645, 1998.
13. Takahashi K, Murakami T, Kamata A, Yumoto R, Higashi Y and Yata N, Pharmacokinetic analysis of the absorption enhancing action of decanoic acid and its derivatives in rats. *Pharm Res* **11:** 388–392, 1994.
14. Cersosimo RJ and Hong WK, Epirubicin: A review of the pharmacology, clinical activity, and adverse effects of an adriamycin analogue. *J Clin Oncol* **4:** 425–439, 1986.
15. Plosker GL and Faulds D, Epirubicin: A review of its pharmacodynamic and pharmacokinetic properties, and therapeutic use in cancer chemotherapy. *Drugs* **45:** 788–856, 1993.
16. Huet S, Marie JP, Gualde N and Robert J, Reference method for detection of Pgp mediated multidrug resistance in human hematological malignancies: A method validated by the laboratories of the French Drug Resistance Network. *Cytometry* **34:** 248–256, 1998.
17. Lo YL, Hsu CY and Huang JD, Use of pharmaceutical excipient Cremophor EL for enhancing intracellular accumu-

lation of epirubicin in Caco-2 cell line. *Chia Nan Annu Bull* **23:** 18–24, 1997.
18. Lo YL, Hsu CY and Huang JD, Comparison of effects of surfactants with other MDR reversing agents on intracellular uptake of epirubicin in Caco-2 cell line. *Anticancer Res* **18:** 3005–3009, 1998.
19. Chantret I, Barbat A, Dussaulx E, Brattain MG and Zweibaum A, Epithelial polarity, villin expression, and enterocytic differentiation of cultured human colon carcinoma cells: A survey of twenty cell lines. *Cancer Res* **48:** 1936–1942, 1988.
20. Hidalgo IJ, Raub TJ and Borchardt RT, Characterization of the human colon carcinoma cell line (Caco-2) as a model system for intestinal epithelial permeability. *Gastroenterology* **96:** 736–749, 1989.
21. Ho NF, Burton PS, Conradi RA and Barsuhn CL, A biophysical model of passive and polarized active transport processes in Caco-2 cells: Approaches to uncoupling apical and basolateral membrane events in the intact cell. *J Pharm Sci* **84:** 21–27, 1995.
22. Hunter J, Hirst BH and Simmons NL, Drug absorption limited by P-glycoprotein-mediated secretory drug transport in human intestinal epithelial Caco-2 cell layers. *Pharm Res* **10:** 743–749, 1993.
23. Hunter J, Jepson MA, Tsuruo T, Simmons NL and Hirst BH, Functional expression of P-glycoprotein in apical membranes of human intestinal Caco-2 cells. Kinetics of vinblastine secretion and interaction with modulators. *J Biol Chem* **268:** 14991–14997, 1993.
24. Walter E, Kissel T and Raddatz P, Transport of peptidomimetic renin inhibitors across monolayers of a human intestinal cell line (Caco-2): Evidence for self-enhancement of paracellular transport route. *Pharm Res* **12:** 1801–1805, 1995.
25. Hosoya KI, Kim KJ and Lee VHL, Age-dependent expression of P-glycoprotein gp170 in Caco-2 cell monolayers, *Pharm Res* **13:** 885–890, 1996.
26. Lo YL, Hsu LR, Song CL, Tsaz YF, Chen CM and Wu DH, Use of Cremophor EL as a MDR reversing agent to enhance intestinal absorption of epirubicin in everted gut sacs of rats and human intestinal epithelial Caco-2 cell layers. *Chia Nan Annu Bull* **24:** 142–149, 1998.
27. Leu BL and Huang JD, Inhibition of intestinal P-glycoprotein and effects on etoposide absorption. *Cancer Chemother Pharmacol* **35:** 432–436, 1995.
28. Sasaki I, Tanaka K, Fujita T, Murakami M, Yamamoto A and Muranishi S, Intestinal absorption of azetirelin, a new thyrotropin-releasing hormone (TRH) analogue. II. *In situ* and *in vitro* absorption characteristics of azetirelin from the rat intestine. *Biol Pharm Bull* **18:** 976–979, 1995.
29. Hu OY, Chang SP, Jame JM and Chen KY, Pharmacokinetic and pharmacodynamic studies with 4′-epi-doxorubicin in nasopharyngeal carcinoma patients. *Cancer Chemother Pharmacol* **24:** 332–337, 1989.
30. Jakobsen P, Sorensen B and Rose C, The pharmacokinetics of high-dose epirubicin and of the cardioprotector ADR-529 given together with cyclophosphamide, 5-fluorouracil, and tamoxifen in metastatic breast-cancer patients. *Cancer Chemother Pharmacol* **35:** 45–52, 1994.
31. Muranishi M, Absorption enhancers.*Crit Rev Ther Drug Carrier Syst* **7:** 1–33, 1990.
32. Tomita M, Hayashi M, Horie T, Ishizawa T and Awazu S, Enhancement of colonic drug absorption by the transcellular permeation route. *Pharm Res* **5:** 786–789, 1988.
33. Tomita M, Hayashi M and Awazu S, Absorption-enhancing mechanism of sodium caprate and decanoylcarnitine in Caco-2 cells. *J Pharmacol Exp Ther* **272:** 739–743, 1995.
34. Takahashi H, Shibasaki T, Takeshita K, Kaiho F and Hayashi M, The enhancing mechanism of capric acid (C10) from a suppository on rectal drug absorption through a paracellular pathway. *Biol Pharm Bull* **20:** 446–448, 1997.
35. Nishimura K, Nozaki Y, Yoshimi A, Nakamura S, Kitagawa M, Kakeya N and Kitao K, Studies on the promoting effects of carboxylic acid derivatives on the rectal absorption of β-lactam antibiotics in rats. *Chem Pharm Bull (Tokyo)* **33:** 282–291, 1985.
36. Guarini S, Fano RA, Rompianesi E, Martinelli AM and Ferrari W, The effect of sodium deoxycholate given by gavage with heparin on the histology of the intestinal mucosa of the rat. *J Pharm Pharmacol* **38:** 922–924, 1986.
37. Lin YJ and Shen WC, Effects of deoxycholate on the transepithelial transport of sucrose and horseradish peroxidase in filter-grown Madin-Darby canine kidney (MDCK) cells. *Pharm Res* **8:** 498–501, 1991.
38. Sawada T, Ogawa T, Tomita M, Hayashi M and Awazu S, Role of paracellular pathway in nonelectrolyte permeation across rat colon epithelium enhanced by sodium caprate and sodium caprylate. *Pharm Res* **8:** 1365–1371, 1991.
39. Wacher VJ, Salphati L and Benet LZ, Active secretion and enterocytic drug metabolism barriers to drug absorption. *Adv Drug Deliv Rev* **20:** 99–112, 1996.
40. Noach ABJ, Roosemalen MCM, Kurosaki Y and Breimer DD, Effect of apical and/or basolateral application of EDTA on the permeability of hydrophilic compounds in a human intestinal epithelial cell line (Caco-2). *J Control Rel* **21:** 206–207, 1992.
41. Anderberg EK, Lindmark T and Artursson P, Sodium caprate elicits dilations in human intestinal tight junctions and enhances drug absorption by the paracellular route. *Pharm Res* **10:** 857–864, 1993.