Effect of Cyclosporin Analogues and FK506 on Transcellular Transport of Daunorubicin and Vinblastine via P-glycoprotein

Kumiko Tanaka,[1] Midori Hirai,[1,3] Yusuke Tanigawara,[1,4] Masato Yasuhara,[1,5] Ryohei Hori,[1,6] Kazumitsu Ueda,[2] and Ken-ichi Inui[1,7]

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Purpose. P-glycoprotein-mediated transcellular transport of anticancer agents and the inhibitory effect of cyclosporin analogs and FK506 were investigated.

Methods. The transcellular transport of daunorubicin and vinblastine by monolayers of LLC-GA5-COL150 cells which overexpressed P-glycoprotein was measured in the presence and absence of cyclosporins or FK506.

Results. Cyclosporins and FK506 inhibited P-glycoprotein-mediated transport of daunorubicin and vinblastine in the order of cyclosporin D, dihydrocyclosporin D > cyclosporin A > FK506 > cyclosporin C, dihydrocyclosporin C. The intracellular accumulation of the anticancer agents was highly associated with the transporting function of P-glycoprotein. The inhibitory effect of cyclosporin D was concentration-dependent. The inhibitory effect of the modulators on P-glycoprotein was not correlated with the immunosuppressive activity, but was correlated with their lipophilicity.

Conclusions. In the transcellular transport system, lipophilicity may be one of the determinants for the inhibitory effect of various multidrug resistance modulators on the P-glycoprotein-mediated transport.

KEY WORDS: P-glycoprotein; cyclosporins; FK506; daunorubicin; vinblastine.

INTRODUCTION

Resistance to anticancer agents is a major problem in cancer chemotherapy. P-glycoprotein is an important component in rendering tumor cells resistant to various anticancer agents. It acts as an ATP-dependent efflux pump to export anticancer agents and lowers their intracellular concentration to sublethal levels (1,2). Various compounds with different structures modulate multidrug resistance by inhibiting P-glycoprotein-mediated efflux of anticancer agents, but the specific structure or chemical group required for the modulators is still unclear. Many of the modulators are known to possess aromatic rings or cationic charge. In our previous study cepharanthin, a biscoclaurine alkaloid which is cationic, inhibited P-glycoprotein-mediated daunorubicin transport (3). However some of the potent modulators do not seem to possess these properties. Cyclosporin A, a cyclic peptide of 11 amino acids, possesses neither aromatic ring nor cationic charge, but is a potent modulator of multidrug resistance (4,5). FK506, a macrocyclic lactone, is also a modulator of multidrug resistance (6). To obtain more information about the structure-activity relationship of the inhibitory potency against P-glycoprotein-associated transport of anticancer agents, we examined the effects of cyclosporin analogues and FK506 on the P-glycoprotein-mediated transport of two anticancer agents, daunorubicin and vinblastine, by measuring the transporting activity of P-glycoprotein directly utilizing the transcellular transport system, and discussed some factors that influence the inhibitory effects of the immunosuppressants.

MATERIALS AND METHODS

Materials

[^{3}H]Daunorubicin (162.8 GBq/mmol) and [methoxy-^{14}C]inulin (0.61 GBq/mmol) were purchased from Du Pont-New England Nuclear (Boston, MA). [^{3}H]Vinblastine sulphate (307 GBq/mmol) was from Amersham International, plc (Buckinghamshire, U.K.). Cyclosporins A, C, D and dihydrocyclosporins C and D were kindly supplied by Sandoz Pharmaceutical (Bazel, Switzerland), and FK506 was a gift from Fujisawa Pharmaceutical Co. (Osaka, Japan) (Fig. 1). All other chemicals were of the highest purity available.

Cell Lines, Transcellular Transport, and Intracellular Accumulation

LLC-PK$_1$ cells, derived from porcine kidney, were transfected with human *MDR1* cDNA, and cells designated LLC-GA5-COL150 were isolated by culturing in the presence of 150 ng/ml colchicine (7).

Transcellular transport and intracellular accumulation study of 100 nM [^{3}H]daunorubicin and 100 nM [^{3}H]vinblastine was done as described previously, using microporous polycarbonate membrane filters (3.0 μM pore size) inside a Transwell™ cell culture chamber (Costar, Cambridge, MA) (3). To examine the effect of cyclosporins and FK506 on the transcellular transport and the intracellular accumulation of anticancer agents, cyclosporins and FK506 were added to both sides of the monolayers 1 hr before the start of the experiments.

Thin Layer Chromatography (TLC) Analysis of Cyclosporins and FK506

Relative hydrophobicity of cyclosporins and FK506 was estimated by TLC analysis. Cyclosporins and FK506 were dissolved in methanol, spotted on Silica Gel TLC plates, and run in a solvent system of chloroform:methanol (94:6). All agents were detected by UV irradiation and the Rf values were measured.

[1] Department of Pharmacy, Kyoto University Hospital, Faculty of Medicine, Kyoto University, Kyoto 606-01, Japan.
[2] Laboratory of Biochemistry, Department of Agricultural Chemistry, Faculty of Agriculture, Kyoto University, Kyoto 606-01, Japan.
[3] Present address: Department of Pharmaceutics, Kobe Pharmaceutical University, Higashinada-ku, Kobe 658, Japan.
[4] Present address: Department of Pharmacy, Kobe University Hospital, Kobe 650, Japan.
[5] Present address: Department of Hospital Pharmacy, School of Medicine, Tokyo Medical and Dental University, Bunkyo-ku, Tokyo 113, Japan.
[6] Present address: Pharmaceutical Research and Technology Institute, Kinki University, 3-4-1 Kowakae, Higashi-osaka 577, Japan.
[7] To whom correspondence should be addressed.

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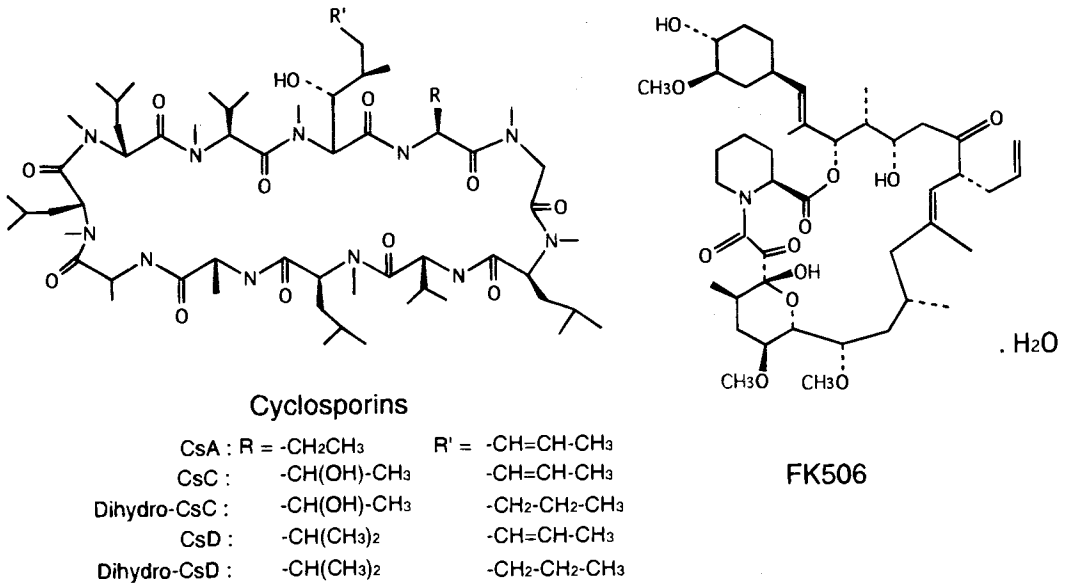

Fig. 1. Chemical structures of cyclosporin analogues and FK506. CsA: cyclosporin A, CsC: cyclosporin C, Dihydro-CsC: dihydrocyclosporin C, CsD: cyclosporin D, Dihydro-CsD: dihydrocyclosporin D.

RESULTS

Transcellular Transport of [^{3}H]Daunorubicin and [^{3}H]Vinblastine in LLC-GA5-COL150 and LLC-PK$_1$ Cells

Since P-glycoprotein overexpressed in LLC-GA5-COL150 cells is localized in the apical membrane (7), a substrate that is expelled by P-glycoprotein would show greater basal-to-apical transport in LLC-GA5-COL150 cells than in LLC-PK$_1$ cells. Figure 2 shows the transcellular transport of [^{3}H]daunorubicin and [^{3}H]vinblastine in LLC-PK$_1$ and LLC-GA5-COL150 cells. With either agent, the basal-to-apical transport in LLC-GA5-COL150 cells greatly exceeded that in LLC-PK$_1$ cells (LLC-GA5-COL150 vs LLC-PK$_1$: 17.7 vs 5.6 pmol/cm^2 in 3 hr for [^{3}H]daunorubicin and 13.1 vs 8.9 pmol/cm^2 in 3 hr for [^{3}H]vinblastine). This phenomenon was accompanied by a marked decrease in intracellular accumulation of the agents in LLC-GA5-COL150 cells, compared to LLC-PK$_1$ cells (LLC-GA5-COL150 vs LLC-PK$_1$: 3.4 vs 54.0 pmol/mg protein in 3 hr for [^{3}H]daunorubicin and 1.2 vs 26.1 pmol/mg protein in 3 hr for [^{3}H]vinblastine).

Effect of Cyclosporin Derivatives and FK506 on Transcellular Transport and Intracellular Accumulation of [^{3}H]Daunorubicin and [^{3}H]Vinblastine

The effect of cyclosporin derivatives and FK506 on [^{3}H]daunorubicin transport was examined in LLC-GA5-COL150 cells (Fig. 3A). Net basal-to-apical transport was calculated by subtracting apical-to-basal transport from basal-to-apical transport, and was used as an index for the P-glycoprotein-mediated transport. The addition of cyclosporin A (5 μM) resulted in a dramatic decrease in net basal-to-apical transport. Cyclosporin D and dihydrocyclosporin D were more effective than cyclosporin A. FK506 had a moderate effect. In contrast, cyclosporin C and dihydrocyclosporin C showed no effect on the transport in either direction. Similar results were also obtained for [^{3}H]vinblastine transport (Fig. 3C).

The effect of these agents on intracellular accumulation of the anticancer agents was examined simultaneously (Fig. 3B, D). Cyclosporin D and dihydrocyclosporin D increased the accumulation extensively to the level of LLC-PK$_1$, and cyclosporin A also increased the intracellular level of the agents. The effect of FK506 was smaller than that of cyclosporin A. On the other hand, cyclosporin C and dihydrocyclosporin C increased the accumulation only slightly.

Relationship Between Lipophilicity and the Effect on P-glycoprotein-mediated Transport of [^{3}H]Daunorubicin

The effect of cyclosporins and FK506 on net basal-to-apical transport of [^{3}H]daunorubicin was plotted against the Rf values determined by TLC according to the procedure described in the methods, and fitted by linear-regression analysis. As shown in Fig. 4, a linear relationship was obtained with a regression coefficient of 0.966. Similar plot was obtained for [^{3}H]vinblastine transport (data not shown).

Concentration Dependence of Cyclosporin D Against Transcellular Transport and Intracellular Accumulation of [^{3}H]Daunorubicin

The concentration dependence of the inhibitory effect of cyclosporin D on [^{3}H]daunorubicin transport and accumulation was examined in LLC-GA5-COL150 cells (Fig. 5). Transcellular transport of [^{3}H]daunorubicin was not affected by 1 μM cyclosporin D. At 3 μM, cyclosporin D had an inhibitory effect, and at 5 μM, it showed strong inhibitory activity; the directional transport of [^{3}H]daunorubicin disappeared. Moreover, cyclosporin D greatly increased [^{3}H]daunorubicin accumulation at 3 μM although only slightly at 1 μM. The effect of

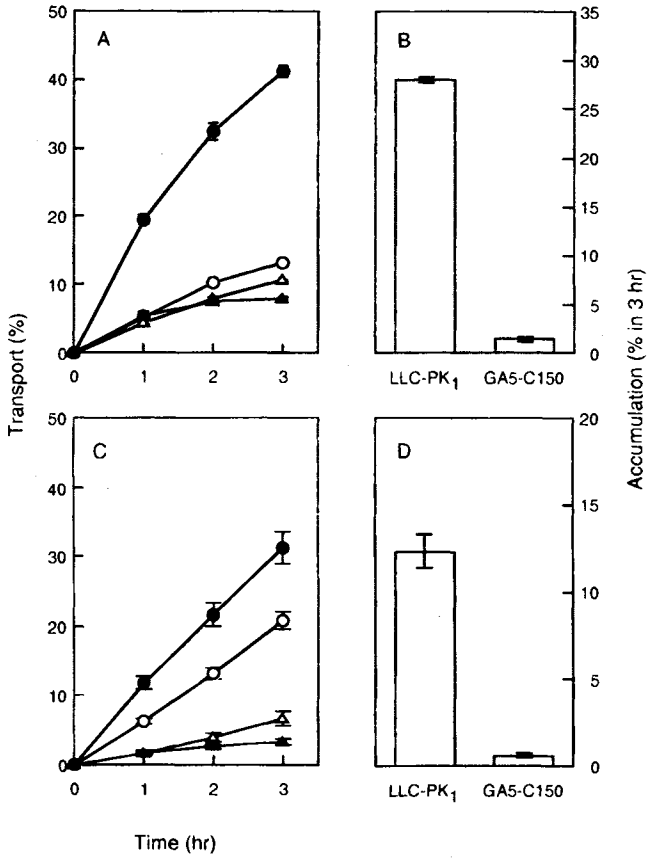

Fig. 2. Transcellular transport (A, C) and intracellular accumulation (B, D) of [^{3}H]daunorubicin and [^{3}H]vinblastine via P-glycoprotein. Transcellular transport of 100 nM [^{3}H]daunorubicin (A) and 100 nM [^{3}H]vinblastine (C) in LLC-PK$_1$ (open symbols) and LLC-GA5-COL150 (solid symbols). The circles (○,●) show the transport from basal to apical side, and the triangles (△,▲) show the transport from apical to basal side. Intracellular accumulation of 100 nM [^{3}H]daunorubicin (B) and 100 nM [^{3}H]vinblastine (D) in LLC-PK$_1$ and LLC-GA5-COL150 cells. The radiolabeled agents were added to the basal side. Similar results were observed when the agents were added to the apical side. GA5-C150:LLC-GA5-COL150. Each point or column represents the mean ± SE of at least three independent experiments.

cyclosporin D at 3 μM resembled that at 5 μM. The inhibitory effect of cyclosporin D on [^{3}H]vinblastine transport was also concentration dependent (data not shown).

DISCUSSION

Cyclosporin A is an immunosuppressive agent that is often used to prevent tissue rejection in organ transplantation. It makes a complex with intracellular receptors in cytosol, cyclophilin, and binds to calcineurin, resulting in inhibition of early events in T cell activation (8). The inhibition of T cell activation by the cyclosporin analogs corresponds to the binding activity against cyclophilin, in the decreasing order of cyclosporin A > cyclosporin C > cyclosporin D (9). In this study the inhibitory effect of cyclosporins on the directional transport of anticancer agents via P-glycoprotein was in the decreasing order of cyclosporin D, dihydrocyclosporin D > cyclosporin A > cyclosporin C, dihydrocyclosporin C; the effect did not correlate with immunosuppressive activity, but clearly correlated with their lipophilicity, as shown in Fig. 5. These findings confirm that the lipophilicity of agents was a determinant of the inhibitory effect on the P-glycoprotein-mediated transport of anticancer agents.

FK506, another immunosuppressive agent with activity 100-fold that of cyclosporin A, also binds to calcineurin as a complex with intracellular receptors, and inhibits T cell activation (10). It also exerted an inhibitory effect on P-glycoprotein-mediated transport, but was much weaker than that of cyclosporin A in spite of its strong immunosuppressive activity (Fig. 3).

Cyclosporin A and FK506 inhibit the photolabeling of P-glycoprotein, and are transported via P-glycoprotein in the transcellular transport system (11–14). Therefore, both cyclosporin analogues and FK506 may inhibit the P-glycoprotein-mediated transport by acting together directly on the protein. However, FK506 stimulates the P-glycoprotein-ATPase activity in the nanomolar range, while cyclosporin A and dihydrocyclosporin C do not stimulate the P-glycoprotein-ATPase activity but act as strong competitive inhibitors of verapamil-stimulated P-glycoprotein-ATPase activity (15). In spite of this difference in mode of action, it is quite interesting that the correlation between the lipophilicity and inhibitory effect on P-glycoprotein is the same for the cyclosporins and FK506 (Fig. 5).

In the present study the amount of [^{3}H]daunorubicin or [^{3}H]vinblastine transported directionally from basal to apical side was inversely related with the intracellular accumulation of the agents. The transcellular transport of [^{3}H]daunorubicin and [^{3}H]vinblastine from basal to apical side increased in LLC-GA5-COL150 cells compared to that in LLC-PK$_1$ cells, while the intracellular accumulation decreased, and in LLC-GA5-COL150 cells the decrease of the unidirectional transport of [^{3}H]daunorubicin or [^{3}H]vinblastine in the presence of inhibitors corresponded well with the increase of intracellular accumulation of these anticancer agents. These findings indicated that the transporting function of P-glycoprotein is a major determinant of the intracellular accumulation of the anticancer agents.

[^{3}H]Vinblastine showed a unidirectional transport in LLC-PK$_1$ cells, in contrast to [^{3}H]daunorubicin transport, which did not show unidirectional transport (Fig. 2). Moreover, in LLC-GA5-COL150 cells, the net basal-to-apical transport of [^{3}H]vinblastine was decreased by only half in the presence of 5 μM cyclosporin D, in contrast to the net transport of [^{3}H]daunorubicin, which was almost completely inhibited by 5 μM cyclosporin D (Fig. 3). Therefore, some mechanisms other than P-glycoprotein may exist for vinblastine transport in LLC-PK$_1$ cells.

In conclusion, our findings suggest that at the cellular level the lipophilicity may be one of the major determinants for the inhibitory effect of various multidrug resistance modulators on the P-glycoprotein-mediated transport.

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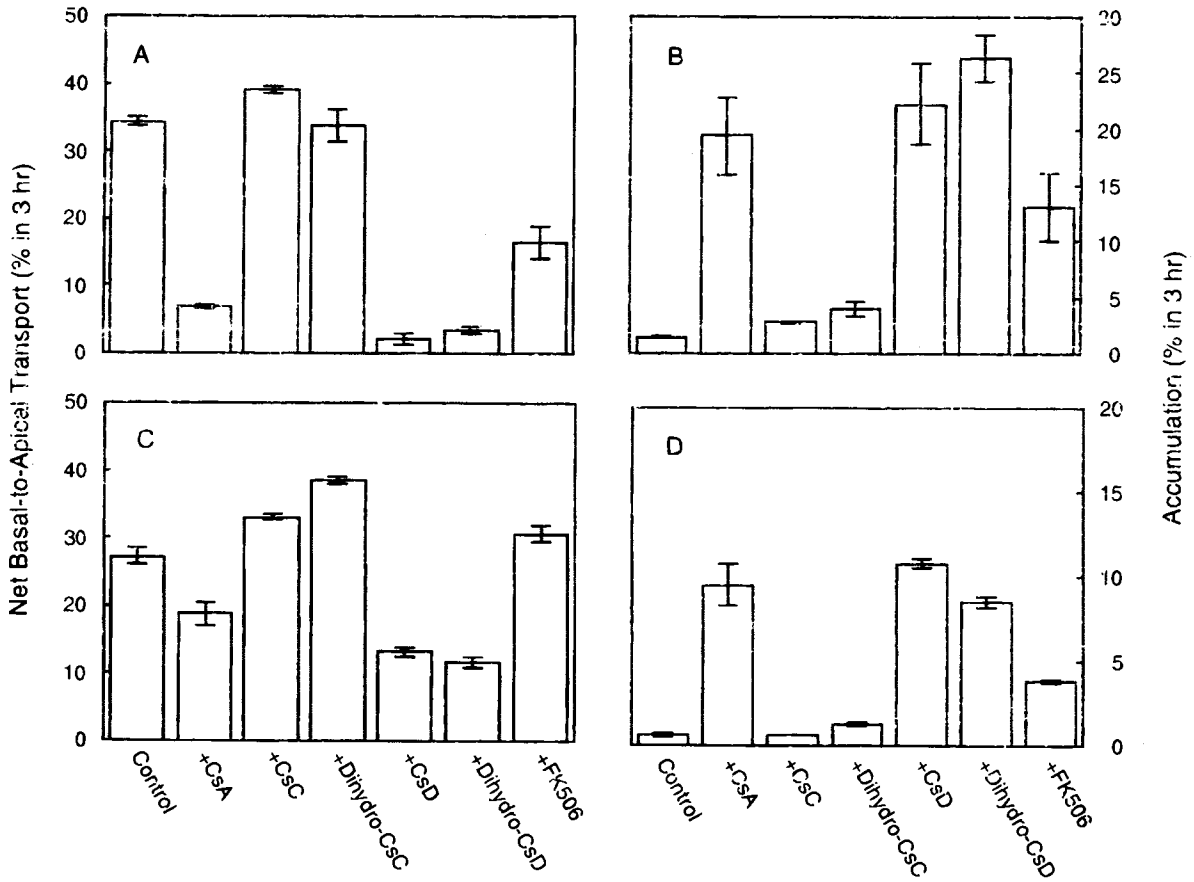

Fig. 3. Effect of cyclosporin analogues and FK506 on transcellular transport (A, C) and intracellular accumulation (B, D) of [^{3}H]daunorubicin and [^{3}H]vinblastine in LLC-GA5-COL150 cells. Transcellular transport of 100 nM [^{3}H]daunorubicin (A) and 100 nM [^{3}H]vinblastine (C). Net basal-to-apical transport was calculated by subtracting the percentage of the radiolabeled agent transported from apical to basal side from that from basal to apical side. Intracellular accumulation of 100 nM [^{3}H]daunorubicin (B) and 100 nM [^{3}H]vinblastine (D). Cyclosporins and FK506 were added at the final concentration of 5 μM. CsA: cyclosporin A, CsC: cyclosporin C, Dihydro-CsC: dihydrocyclosporin C, CsD: cyclosporin D, Dihydro-CsD: dihydrocyclosporin D. Each column represents the mean ± SE of at least three independent experiments.

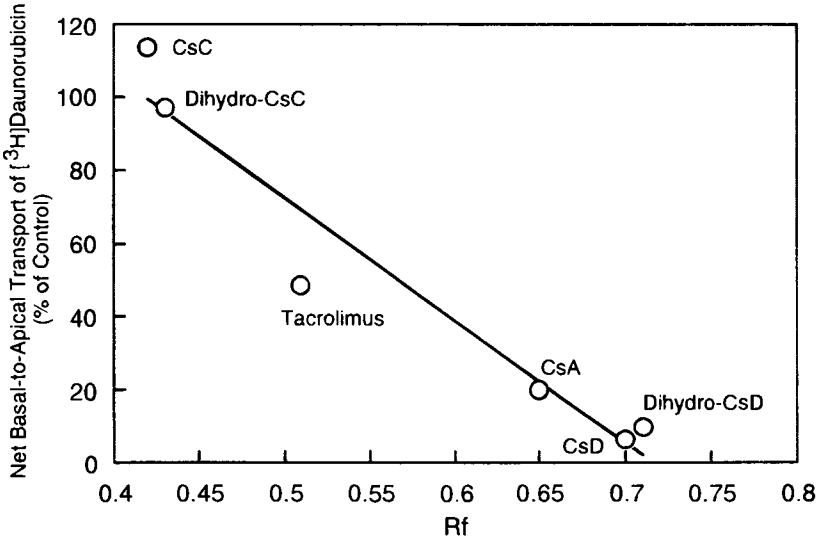

Fig. 4. Relationship between Rf values and the effect on net basal-to-apical transport of [^{3}H]daunorubicin for cyclosporins and FK506 in LLC-GA5-COL150 cells. Rf values were measured as described in Methods. The data for the effect on net basal-to-apical transport of 100 nM [^{3}H]daunorubicin are shown as % of control. Correlation coefficient of linear regression analysis was 0.966.

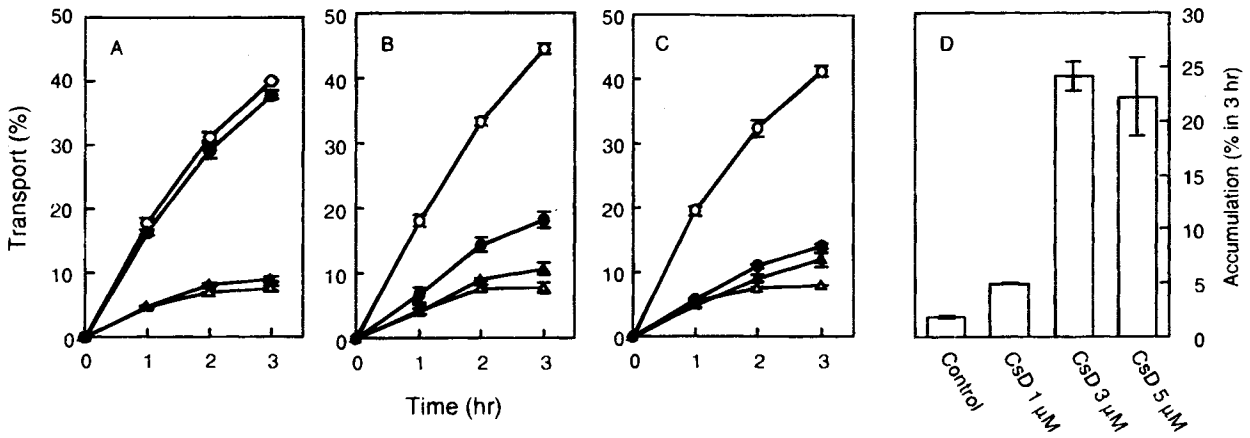

Fig. 5. Inhibitory activity of various concentrations of cyclosporin D on transcellular transport (A–C) and intracellular accumulation (D) of 100 nM [^{3}H]daunorubicin in LLC-GA5-COL150 cells. Effect of 1 μM (A); 3 μM (B); 5 μM (C) of cyclosporin D (CsD) on transcellular transport of [^{3}H]daunorubicin in LLC-GA5-COL150 cells. Transport from basal to apical side (○,●) and that from apical to basal side (△,▲) were examined in the absence (open symbols) or in the presence (solid symbols) of cyclosporin D. Intracellular accumulation (D) of [^{3}H]daunorubicin in the absence or in the presence of cyclosporin D. Each point or column represents the mean ± SE of at least three independent experiments.

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