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Inhibitory Effects of a Cyclosporin Derivative, SDZ PSC 833, on Transport of Doxorubicin and Vinblastine via Human P-Glycoprotein

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The inhibitory effects of SDZ PSC 833 (PSC833), a non-immunosuppressive cyclosporin derivative, on the P-glycoprotein (P-gp)-mediated transport of doxorubicin and vinblastine were compared with those of cyclosporin A (Cs-A). The transcellular transport of the anticancer drugs and PSC833 across a monolayer of LLC-GA5-COL150 cells, which overexpress human P-gp, was measured. Both PSC833 and Cs-A inhibited P-gp-mediated transport of doxorubicin and vinblastine in a concentration-dependent manner and increased the intracellular accumulation of doxorubicin and vinblastine in LLC-GA5-COL150 cells. The values of the 50%-inhibitory concentration (IC_{50}) of PSC833 and Cs-A for doxorubicin transport were 0.29 and 3.66 μM, respectively, and those for vinblastine transport were 1.06 and 5.10 μM, respectively. The IC_{50} of PSC833 for doxorubicin transport was about 4-fold less than that for vinblastine transport, suggesting that the combination of PSC833 and doxorubicin might be effective. PSC833 itself was not transported by P-gp and had higher lipophilicity than Cs-A. These results indicated that the inhibitory effect of PSC833 on P-gp-mediated transport was 5- to 10-fold more potent than that of Cs-A, and this higher inhibitory effect of PSC833 may be related to the absence of PSC833 transport by P-gp and to the higher lipophilicity of PSC833.

Key words: SDZ PSC 833 — Cyclosporin A — P-glycoprotein — Multidrug resistance

Multidrug resistance (MDR) is a major obstacle in cancer chemotherapy. P-Glycoprotein (P-gp), a plasma membrane protein, actively transports drugs out of the cells and decreases their intracellular accumulation, resulting in resistance to multiple anticancer drugs.[1, 2] Overexpression of P-gp is closely associated with MDR, and the blocking of P-gp-mediated transport is known to reverse MDR. Blocking agents are generally called MDR modulators, and the reversal of resistance by MDR modulators is expected to improve the outcome of cancer chemotherapy.[3]

SDZ PSC 833 (PSC833), a new analog of cyclosporin D, was found to be a potent MDR-modulating agent.[4, 5] Cyclosporin A (Cs-A) also shows MDR-modulating activity,[6, 7] but its immunosuppressive effect and nephrotoxicity limit its clinical usefulness for reversal of MDR. On the other hand, PSC833 lacks nephrotoxic and hemodynamic side effects, as well as immunosuppressive activity. Clinical trials using PSC833 combined with anticancer drugs (doxorubicin, etoposide etc.) to circumvent MDR are currently under way.[3, 8–13]

Previous *in vitro* studies have evaluated the efficacy of PSC833 for the reversal of MDR using the cell growth inhibition assay.[4, 5, 14–20] They also measured uptake, efflux and accumulation of drugs to evaluate the transport activity.[14, 15, 18–21] However, highly lipophilic compounds such as PSC833 and Cs-A are readily adsorbed on the plasma membranes in a non-specific fashion, so it was difficult to evaluate quantitatively the P-gp-mediated transport by means of the previous methods. In this study, we employed a novel experimental system using monolayers of LLC-GA5-COL150 cells that express human P-gp as a result of transfection with human *MDR1* cDNA.[22, 23] This transcellular transport system is suitable for direct assessment of the amount that is actually pumped out of the cells by P-gp without the need to consider adsorption on cellular components.[22–29]

In this study, we compared PSC833 and Cs-A with respect to the inhibitory effects on P-gp-mediated transcellular transport of [^{14}C]doxorubicin and [^{3}H]vinblastine. Possible reasons for the difference in potency are discussed.

MATERIALS AND METHODS

Chemicals [^{14}C]Doxorubicin (2.11 GBq/mmol), [^{3}H]vinblastine (422 GBq/mmol), [methoxy-^{14}C]inulin (308 MBq/

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mmol) and [^{3}H]inulin (25.2 GBq/mmol) were obtained from Amersham International, plc. (Buckinghamshire, UK). Vinblastine sulfate and colchicine were purchased from Wako Pure Chemical Industries, Ltd. (Osaka). PSC833 and Cs-A were kindly supplied by Novartis Pharma (Basel, Switzerland). All other chemicals were of the highest purity available.

Cell culture LLC-PK$_1$ cells, derived from porcine kidney, and LLC-GA5-COL150 cells established by transfection of human *MDR1* cDNA into LLC-PK$_1$ cells,[22, 23] were maintained by serial passages in plastic culture dishes (Iwaki Glass, Chiba). Cells were incubated in complete medium consisting of Medium199 (Dainippon Pharmaceutical Co., Ltd., Osaka) with 10% fetal bovine serum (BioWhittaker, Walkersville, MD) and 150 ng/ml of colchicine for LLC-GA5-COL150 cells without antibiotics. LLC-PK$_1$ and LLC-GA5-COL150 cells (1×10^6 and 1.5×10^6 cells, respectively) were seeded in plastic dishes (100 mm diameter) in 10 ml of complete culture medium. Monolayer cultures were grown in an atmosphere of 5% CO_2-95% air at 37°C, and were subcultured every 4 and 7 days for LLC-PK$_1$ and LLC-GA5-COL150 cells, respectively, with 0.02% EDTA and 0.05% trypsin (Gibco BRL Life Technologies, Inc., Grand Island, NY).

Transcellular transport and intracellular accumulation of [^{14}C]doxorubicin LLC-PK$_1$ and LLC-GA5-COL150 cells were seeded on microporous polycarbonate membrane filters (3.0 μm pore size, inside diameter 24.5 mm, Transwell™ 3414, Costar, Cambridge, MA) at a cell density of 4×10^5 and 5×10^5 cells/cm^2, respectively. Cells were cultured in 2.6 and 1.5 ml of complete culture medium in the outside and inside of the chamber, respectively, in an atmosphere of 5% CO_2-95% air at 37°C for 3 days. The experiments were performed using the procedure described previously.[22–29] Four hours before the start of the experiments, all culture media were replaced with fresh medium without colchicine. Transcellular transport experiments were determined using cell monolayers. Medium on the donor side of the monolayers was replaced with 2 ml of fresh medium containing 800 n*M* [^{14}C]doxorubicin (3.4 kBq) together with 220 n*M* [^{3}H]inulin (34 kBq) and that on the receiver side was replaced with 2 ml of fresh medium alone. The monolayers were incubated in 5% CO_2-95% air at 37°C, and aliquots (25 μl) of the incubation medium on the receiver side were collected at 3, 6, 20 and 24 h. The paracellular leakage was estimated in terms of the amount of [^{3}H]inulin appearing on the receiver side. As human P-gp was overexpressed in LLC-GA5-COL150 cells on the apical membrane, substrates transported by P-gp showed greater basal-to-apical transport in LLC-GA5-COL150 than in LLC-PK$_1$ cells.[22, 23]

To examine the inhibitory effects of PSC833 and Cs-A, PSC833 (0.1, 0.5, 1 and 2 μM) or Cs-A (2, 5 and 10 μM) was added to the medium on both sides of the cell monolayers 1 h before adding the radiolabeled drugs. The incubation medium also contained the same concentration of these compounds.

For accumulation studies, immediately after the last sampling, the medium was removed by aspiration, and the monolayers were rapidly washed twice with ice-cold phosphate-buffered saline on each side. The filters with monolayers were detached from the chambers, the cells on the filters were solubilized in 1 ml of 0.3 *N* NaOH overnight, and the radioactivity was measured. Radioactivities of the collected media and solubilized cell monolayers were counted in 3 ml of ACS II (Amersham International) by liquid scintillation counting (Beckman, LS6000TA, Fullerton, CA) and are presented as the percentage fraction of the total radioactivity.

Transcellular transport and intracellular accumulation of [^{3}H]vinblastine In the case of vinblastine, 3 h before the start of the experiments, culture media were replaced with fresh medium without colchicine. Medium on the donor side of the monolayers was replaced with 2 ml of fresh medium containing 100 n*M* [^{3}H]vinblastine (34 kBq) together with 660 n*M* [methoxy-^{14}C]inulin (3.4 kBq) and that on the receiver side was replaced with 2 ml of fresh medium alone. As the transport rate of [^{3}H]vinblastine was faster than that of [^{14}C]doxorubicin, sampling was conducted at 1, 2 and 3 h. Other procedures were the same as those for the [^{14}C]doxorubicin transport experiment.

Transcellular transport of PSC833 Transport of PSC833 by P-gp was studied using the same cell system. All culture media were replaced with fresh medium without colchicine 3 h before the start of the experiments. Medium on the donor side of the monolayers was replaced with 2 ml of fresh medium containing 10 μM PSC833 and that on the receiver side was replaced with fresh medium without PSC833. The monolayers were incubated in 5% CO_2-95% air at 37°C and sampling was carried out at 3 and 6 h. Determination of PSC833 concentrations was performed by HPLC. Aliquots of medium were extracted with 2 ml of 90 m*M* HCl and 5 ml of *tert*-butylmethylether. The organic layers were transferred and extracted with 1 ml of Titrisol buffer (pH 10). Then, the organic layer was transferred to another Teflon tube and evaporated to dryness at 50°C, and the residue was extracted with 300 μl of acetonitrile/water/methanol (50:30:20) and 1 ml of *n*-heptane. The aqueous layers were dissolved in the HPLC mobile phase and 150 μl was injected into the chromatograph. PSC833 was determined using an LC-6A high-performance liquid chromatograph (Shimadzu Co., Kyoto) equipped with an SPD-6AV UV spectrophotometric detector (210 nm, Shimadzu Co.) and an integrator (Chromatopac C-R4A, Shimadzu Co.). The peak areas were used for quantification. The column was

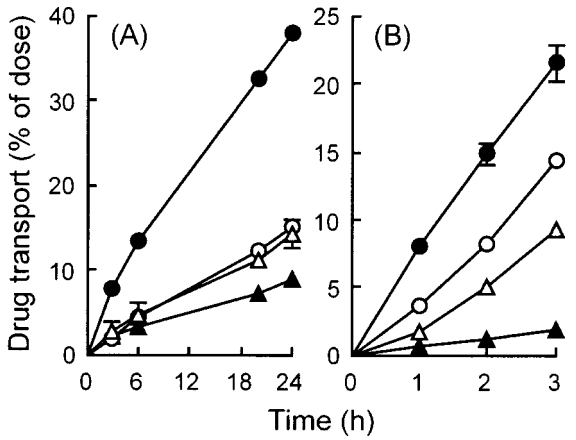

Fig. 1. Transcellular transport of [^{14}C]doxorubicin (A) and [^{3}H]vinblastine (B) across monolayers of LLC-PK$_1$ (open symbols) and LLC-GA5-COL150 (closed symbols) cells. The circles (○, ●) reveal the basal-to-apical transport and the triangles (△, ▲) show the apical-to-basal transport. Each point represents the mean±SE of at least three independent experiments.

a "TSK-GEL" ODS-80TM, 4.6-mm inside diameter×150 mm (Tosoh, Tokyo), with a mobile phase of water/acetonitrile=21:79 at a flow rate of 1.2 ml/min, and the column temperature was maintained at 70°C. The quality of the assay was confirmed to be identical to that of radioimmunoassay using the same samples.

Thin-layer chromatography (TLC) of PSC833 and Cs-A The relative lipophilicities of PSC833 and Cs-A were assessed by TLC.[28] PSC833 and Cs-A were dissolved in methanol, spotted onto silica gel TLC plates (Wako Pure Chemical Industries, Ltd.), and run with a mixture of chloroform and methanol (94:6). The plates were exposed to UV irradiation to detect the bands. The R_f values of PSC833 and Cs-A were 0.831 and 0.812, respectively.

Estimation of 50%-inhibitory concentration (IC_{50}) of PSC833 and Cs-A on [^{14}C]doxorubicin and [^{3}H]vinblastine transport The IC_{50} values of PSC833 and Cs-A for the net basal-to-apical transport of [^{14}C]doxorubicin and [^{3}H]vinblastine were estimated as follows. The net basal-to-apical transport of [^{14}C]doxorubicin and [^{3}H]vin-

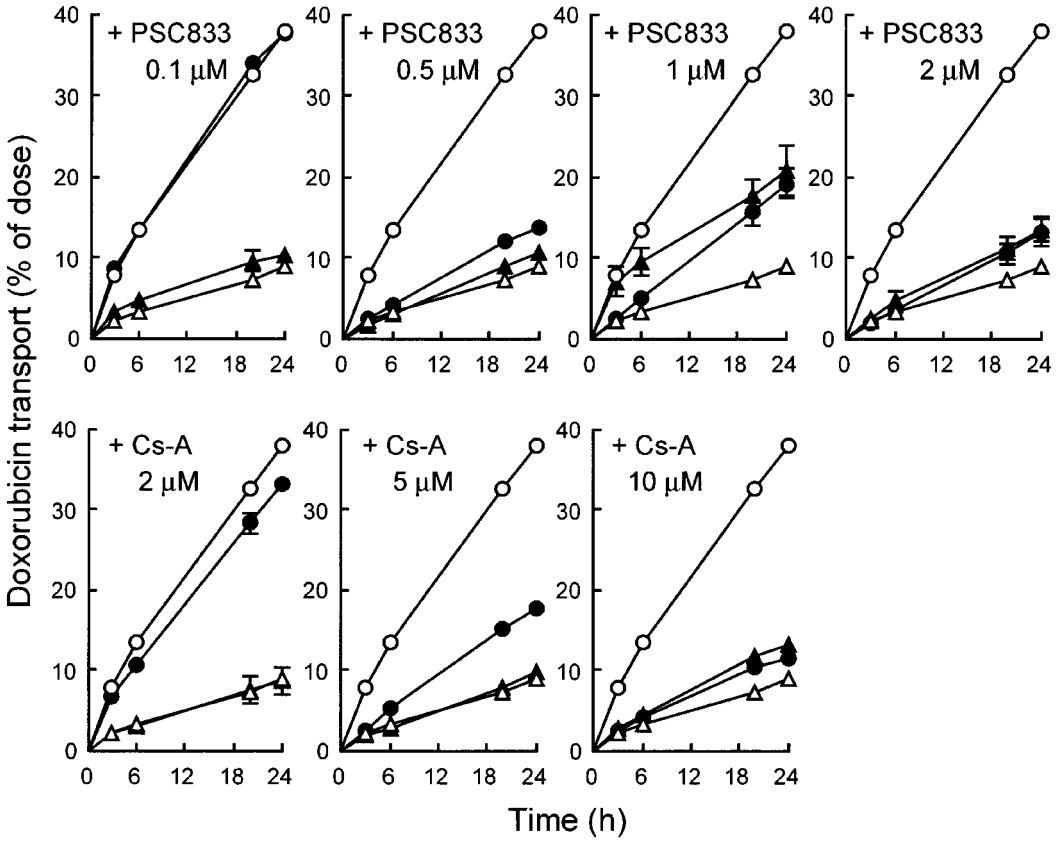

Fig. 2. Inhibitory effects of PSC833 and Cs-A on transcellular transport of [^{14}C]doxorubicin in LLC-GA5-COL150 cells. The basal-to-apical transport (●) and the apical-to-basal transport (▲) in the presence of inhibitors were compared with the same movements in the absence of inhibitors (○, △). Each point represents the mean±SE of at least three independent experiments.

blastine was calculated by subtracting the apical-to-basal transport from the basal-to-apical transport. At several concentrations, PSC833 or Cs-A caused inhibition of the net basal-to-apical transport, shown as the percentage reduction of the net basal-to-apical transport compared to controls. The percentage inhibition of the net basal-to-apical transport was plotted against the concentration of PSC833 or Cs-A, and fitted to the sigmoid E_{max} model (1) by means of a nonlinear least-squares fitting method (Solver, "Microsoft" Excel, version 5.0a).

$$E=E_{max}\times C^r/(IC_{50}{}^r+C^r) \quad (1)$$

where E is the percentage inhibition (%) by PSC833 or Cs-A, E_{max} is the maximum inhibition rate (that is, 100%), r is a constant, and C is the concentration of PSC833 or Cs-A (μM) in the culture media.

RESULTS

Transcellular transport of [^{14}C]doxorubicin and [^{3}H]vinblastine in LLC-PK$_1$ and LLC-GA5-COL150 cells Fig. 1 shows the transcellular transport of [^{14}C]doxorubicin and [^{3}H]vinblastine in LLC-PK$_1$ and LLC-GA5-COL150 cells. The basal-to-apical transport of [^{14}C]doxorubicin in LLC-GA5-COL150 cells greatly exceeded that in LLC-PK$_1$ cells, whereas the apical-to-basal transport decreased (Fig. 1A). Similar results were also obtained for [^{3}H]vinblastine transport (Fig. 1B).

Inhibitory effects of PSC833 and Cs-A on the transcellular transport of [^{14}C]doxorubicin Fig. 2 represents the inhibitory effects of PSC833 and Cs-A on the [^{14}C]doxorubicin transport in LLC-GA5-COL150 cells. PSC833 at 0.1 μM had little inhibitory effect on [^{14}C]doxorubicin transport, but at concentrations greater than 0.5 μM, PSC833 showed a strong inhibitory effect. Cs-A at 2 μM did not inhibit [^{14}C]doxorubicin transport. However, Cs-A at 10 μM caused complete inhibition of [^{14}C]doxorubicin transport.

Fig. 3A shows the effects of PSC833 and Cs-A on the intracellular accumulation of [^{14}C]doxorubicin. The intracellular accumulation of [^{14}C]doxorubicin in LLC-GA5-COL150 cells in the absence of an MDR modulator was reduced as compared with that of LLC-PK$_1$ cells. PSC833 at 0.5 μM completely restored the intracellular accumulation of [^{14}C]doxorubicin to the level in LLC-PK$_1$ cells. In the case of Cs-A, the intracellular accumulation of

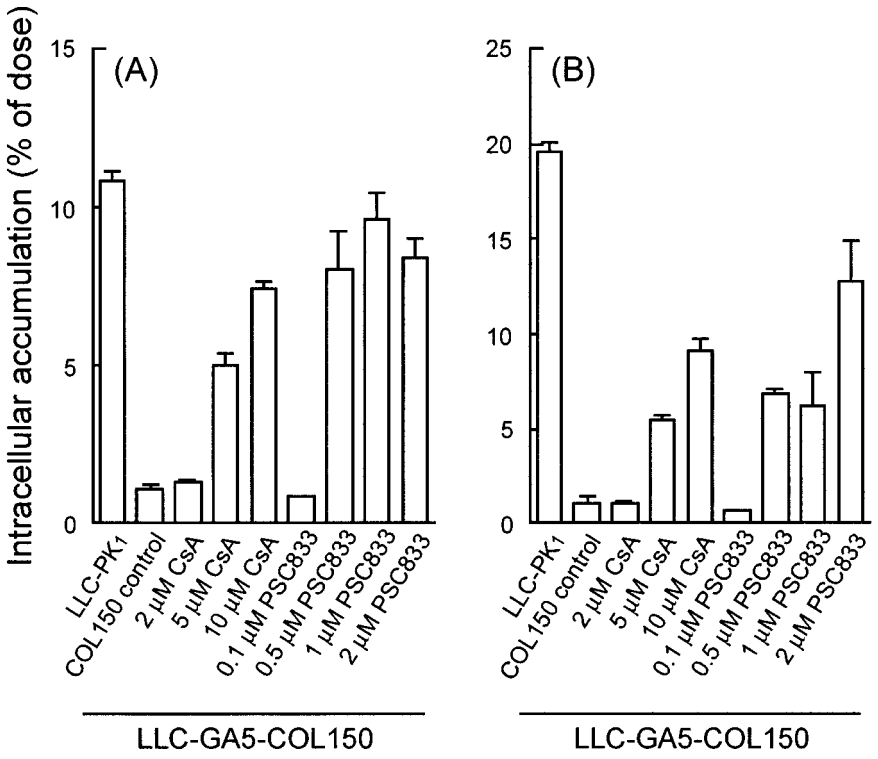

Fig. 3. Intracellular accumulation of [^{14}C]doxorubicin (A) and [^{3}H]vinblastine (B) in LLC-PK$_1$ and LLC-GA5-COL150 cells. The radiolabeled drugs were added to the basal side. Similar results were observed when the drugs were added to the apical side. Each bar represents the

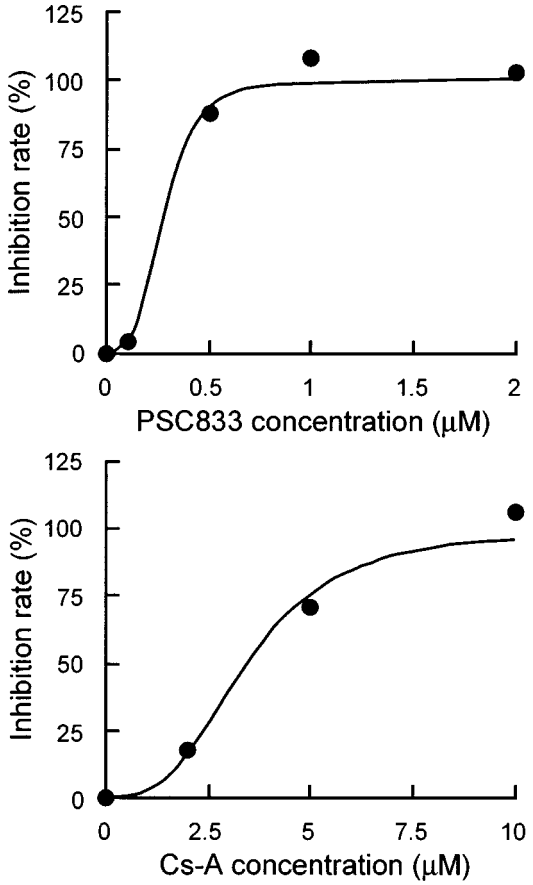

Fig. 4. The concentration vs. inhibitory rate curve of PSC833 and Cs-A for [^{14}C]doxorubicin transport in LLC-GA5-COL150 cells. Closed circles show the observed points and the solid line is that fitted by the sigmoid E_{max} model. Each point represents the mean of at least three independent experiments.

[^{14}C]doxorubicin increased at 5 μM Cs-A, and at 10 μM Cs-A the effect was nearly saturated.

Fig. 4 shows the concentration vs. inhibition rate curve of PSC833 and Cs-A for [^{14}C]doxorubicin transport in LLC-GA5-COL150 cells. PSC833 and Cs-A inhibited [^{14}C]doxorubicin transport in a concentration-dependent manner. The inhibitory effects of PSC833 and Cs-A on [^{14}C]doxorubicin transport were best fitted to the sigmoid E_{max} model. The IC_{50} values estimated from these results are summarized in Table I. The IC_{50} of PSC833 was almost 10-fold less than that of Cs-A for [^{14}C]doxorubicin transport.

Inhibitory effects of PSC833 and Cs-A on the transcellular transport of [^{3}H]vinblastine Fig. 5 shows the effects of PSC833 and Cs-A on [^{3}H]vinblastine transport in LLC-GA5-COL150 cells. As in the case of doxorubicin, both PSC833 and Cs-A inhibited [^{3}H]vinblastine transport in a concentration-dependent manner. [^{3}H]Vinblastine transport was almost completely inhibited in the presence of 2 μM PSC833, and a similar effect was observed at 10 μM Cs-A. The intracellular accumulation of [^{3}H]vinblastine in LLC-GA5-COL150 cells approached the control level in the presence of 2 μM PSC833 (Fig. 3B). However, Cs-A restored the accumulation to only half of the LLC-PK_1 level even at 10 μM.

Table I. Fifty Percent Inhibitory Concentrations of PSC833 and Cs-A for the Transcellular Transport of [^{14}C]Doxorubicin and [^{3}H]Vinblastine in LLC-GA5-COL150 Cells

	[^{14}C]Doxorubicin transport	[^{3}H]Vinblastine transport
PSC833	0.291	1.06
Cs-A	3.66	5.10

Results represent the IC_{50} of PSC833 and Cs-A for net basal-to-apical transport of doxorubicin at 20 h or vinblastine at 3 h. Net basal-to-apical transport was calculated by subtracting the apical-to-basal transport from the basal-to-apical transport. The presence of PSC833 or Cs-A at several concentrations caused inhibition of the net basal-to-apical transport, shown as the percentage reduction in the net basal-to-apical transport from that in the absence of these drugs. The percentage inhibition of the net basal-to-apical transport was plotted against the concentration of PSC833 or Cs-A, and fitted to the sigmoid E_{max} model by means of the nonlinear least-squares fitting method. The IC_{50} values were estimated from the sigmoid E_{max} model.

The concentration vs. inhibition rate curve of PSC833 and Cs-A on [^{3}H]vinblastine transport was also plotted and the results for vinblastine were similar to those for doxorubicin (data not shown). The IC_{50} value for PSC833 was about 5-fold less than that for Cs-A in the [^{3}H]vinblastine transport experiment (Table I). Further, the IC_{50} of PSC833 for [^{14}C]doxorubicin transport was about 4-fold less than that for [^{3}H]vinblastine transport, although the IC_{50} of Cs-A was similar for both drugs.

Transcellular transport of PSC833 Fig. 6 shows the transcellular transport of PSC833 in LLC-PK_1 and LLC-GA5-COL150 cells. The basal-to-apical transport of 10 μM PSC833 in LLC-PK_1 cells was similar to the apical-to-basal transport (Fig. 6A). Similar results were also obtained for LLC-GA5-COL150 cells (Fig. 6B). Furthermore, the transported amount of PSC833 in LLC-GA5-COL150 cells was approximately the same as that in LLC-PK_1 cells.

DISCUSSION

The present methodology allowed us to make a direct assessment of P-gp-mediated drug transport and its inhibition by measuring the amounts that were actually expelled from the cells.

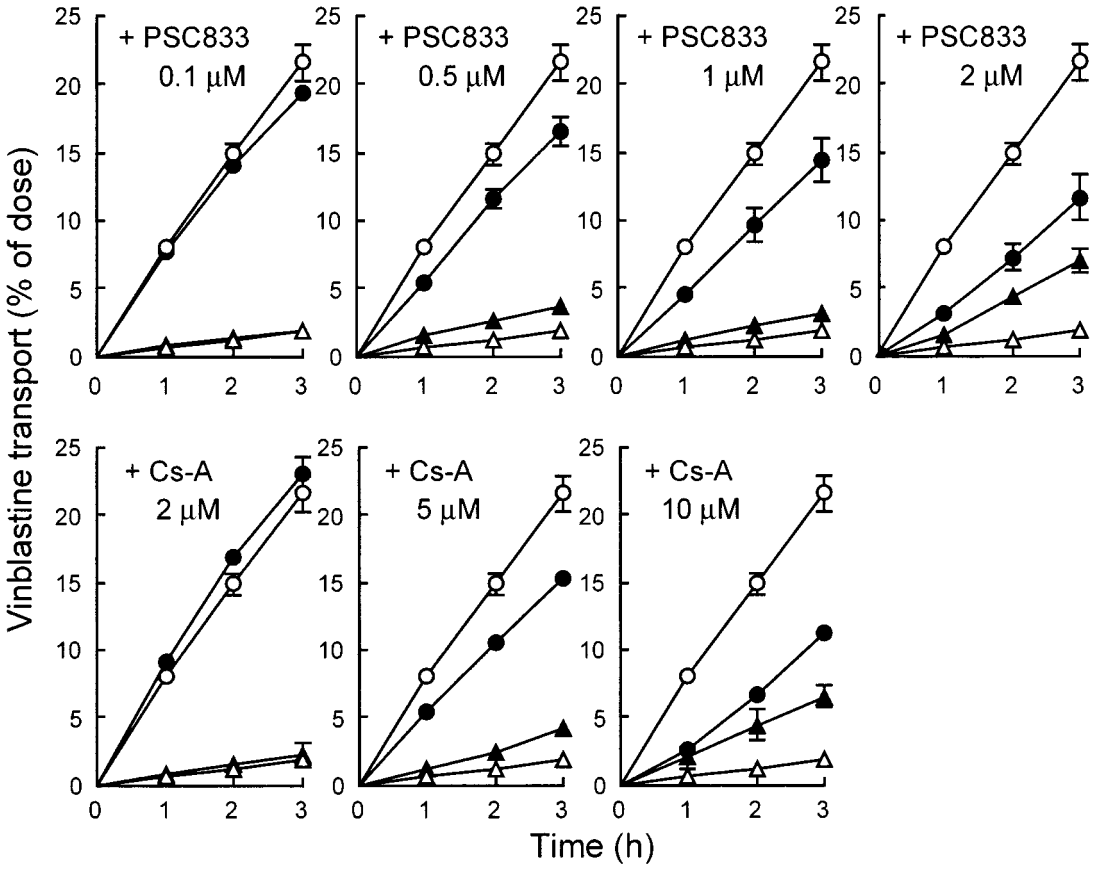

Fig. 5. Inhibitory effects of PSC833 and Cs-A on transcellular transport of [^{3}H]vinblastine in LLC-GA5-COL150 cells. The basal-to-apical transport (●) and the apical-to-basal transport in (▲) the presence of inhibitors were compared with the same movements in the absence of inhibitors (○, △). Each point represents the mean±SE of at least three independent experiments.

The IC_{50} of PSC833 for [^{14}C]doxorubicin transport was 10% of that of Cs-A, and in the case of vinblastine the IC_{50} of PSC833 was 20% of that of Cs-A (Table I). The inhibitory effect of PSC833 on P-gp-mediated transport of the anticancer drugs was about 5- to 10-fold stronger than that of Cs-A. It was demonstrated previously that the concentrations of PSC833 and Cs-A capable of reversing multidrug resistance were 0.03 to 2 *μM* and 0.3 to 10 *μM*, respectively, by cell growth inhibition assay. [4, 5, 14, 18, 20] The rank order of the inhibitory potencies of PSC833 and Cs-A in this study is in good agreement with those in other studies, although the IC_{50} values obtained in this study (Table I) were different from those reported previously. In this study, we measured the P-gp-mediated transport rate within 24 h, whereas in the previous studies the reversal of MDR was examined by cell growth inhibition assay, conducted after 3 days in culture in the presence of the anticancer drugs and the MDR modulator. Furthermore, the cell lines used in the present and those previous studies were different.

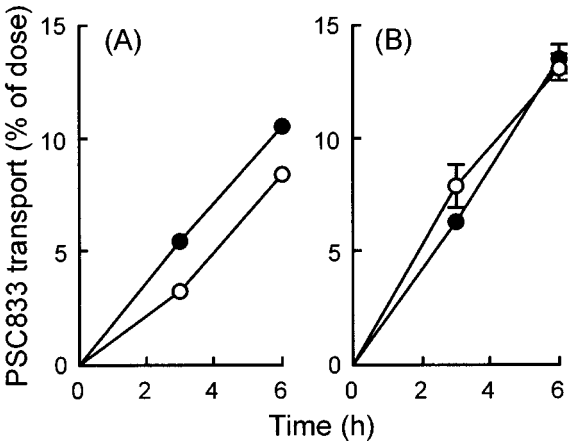

Fig. 6. Transcellular transport of 10 *μM* PSC833 in LLC-PK$_1$ (A) and LLC-GA5-COL150 (B) cells. The open circles (○) indicate the basal-to-apical transport and the closed circles (●) show the apical-to-basal transport. Each point represents the mean±SE of at least three independent experiments.

Lipophilicity is one of the major determinants of the inhibitory effect of various MDR modulators on P-gp-mediated transport at the cellular level.[28] Archinal-Mattheis *et al*. suggested that the higher lipophilicity of PSC833 compared with that of Cs-A can facilitate its transport into cells, and therefore PSC833 can readily gain access to P-gp and enhance its potency.[30] The R_f values (an index of the relative lipophilicity) of PSC833 and Cs-A, obtained by using TLC analysis, were 0.831 and 0.812, respectively. Although the difference in our TLC analysis was not large, it was also reported that PSC833 was more lipophilic than Cs-A based on the partition coefficient.[30] The higher lipophilicity has been considered to be a possible factor responsible for the higher inhibitory effect of PSC833 than that of Cs-A. Other possible reasons to explain the higher activity of PSC833 are discussed below.

The lipophilic MDR modulators can be divided into two types; substrates transported by P-gp, and those which are not transported.[26] We have demonstrated that PSC833 is not transported by P-gp (Fig. 6), but it was reported that Cs-A is transported by P-gp.[24, 25] MDR modulators transported by P-gp are expelled into the extracellular space, resulting in a lower intracellular concentration in P-gp-expressing cells.[25, 29] However, if an MDR modulator is not transported by P-gp, its intracellular concentration would be maintained at high levels even in P-gp-expressing cells.[26] So, it is expected that a high concentration of PSC833 can be maintained in the vicinity of the drug-binding sites of P-gp. Furthermore, PSC833 was approximately 2.5-fold more effective than Cs-A in displacing the photoactivatable cyclosporin analogue,[31] although the drug-binding sites on P-gp are common between PSC833 and Cs-A.[30] Therefore, the absence of PSC833 transport by P-gp and the higher binding activity of PSC833 to P-gp contributed to the higher inhibitory effect of this drug on P-gp-mediated transport.

The inhibitory effect of PSC833 on [^{14}C]doxorubicin transport was about 4-fold stronger than that on [^{3}H]vinblastine transport (Table I). The reason for the difference in the inhibitory effects of PSC833 on [^{14}C]doxorubicin and [^{3}H]vinblastine transport remains unknown, but we speculate that the affinities of the anticancer drugs for P-gp are different. The more effective inhibition of [^{14}C]doxorubicin transport by PSC833 suggests that combination therapy with PSC833 and doxorubicin may be clinically advantageous. In clinical trials, combination therapy with PSC833 and doxorubicin produced an increase in the area under the concentration-time curve (AUC) of doxorubicin by 20–199%[3, 9, 10, 12] and the MDR modulator effect of PSC833 has thus been confirmed.

Several clinical trials of combination therapy with anticancer drugs and MDR modulators have been carried out. Boote *et al*. reported that the target PSC833 concentration was 1000 ng/ml (about 0.8 μM).[11] PSC833 increased the AUC of coadministered anticancer drugs at concentrations of 1000–2000 ng/ml.[3, 8–13] On the other hand, the target concentration of Cs-A to reverse MDR was 3000–4800 ng/ml (about 2.5–4.0 μM),[32] and these concentrations of Cs-A caused an increase in the AUC of coadministered anticancer drugs and reversible hyperbilirubinemia.[32–37] PSC833 was more clinically effective than Cs-A. Therefore, our transcellular transport system is expected to be a useful tool to predict the relative effectiveness of MDR modulators for cancer chemotherapy.

In conclusion, we have demonstrated that the inhibitory effect of PSC833 on P-gp-mediated transport of anticancer drugs is 5- to 10-fold more effective than that of Cs-A. The higher inhibitory effect of PSC833 is considered to be due to the absence of PSC833 transport by P-gp and also to its higher lipophilicity compared with Cs-A.

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