Jpn. J. Cancer Res. **91**, 248–254, February 2000

The Novel Anticancer Drug KRN5500 Interacts with, but is Hardly Transported by, Human P-Glycoprotein

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The interaction of the novel anticancer drug KRN5500, a spicamycin derivative, with human P-glycoprotein (P-gp) was analyzed from the viewpoint of cellular pharmacokinetics, i.e. by means of [^{3}H]azidopine photoaffinity labeling, cellular accumulation and transcellular transport experiments. In this study, P-gp-overexpressing LLC-GA5-COL150 cells, porcine kidney epithelial LLC-PK$_1$ cells transformed with human *MDR1* cDNA, were used, since this cell line constructs monolayers with tight junctions, and would provide sufficient information for analyzing the cellular pharmacokinetics. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay revealed that the growth-inhibitory effect of KRN5500 in LLC-GA5-COL150 cells was comparable to that in LLC-PK$_1$ cells (IC$_{50}$ = 79.4 and 72.7 n*M*, respectively), but the inhibition of [^{3}H]azidopine binding by KRN5500 was concentration-dependent in the membrane fraction of LLC-GA5-COL150 cells. The cellular accumulation of [^{14}C]KRN5500 after its basal application in LLC-GA5-COL150 cells was slightly lower than that in LLC-PK$_1$ cells, and was restored by the multidrug resistance (MDR) modulator SDZ PSC 833. The basal-to-apical transport of [^{14}C]KRN5500 in LLC-GA5-COL150 cells was also slightly higher than that in LLC-PK$_1$ cells, and was inhibited by SDZ PSC 833. However, the basal-to-apical transport of [^{14}C]KRN5500 in LLC-GA5-COL150 cells was only a little higher than the apical-to-basal transport. Consequently, these results demonstrated that KRN5500 interacted with, but was hardly transported via, P-gp. These observations suggested that KRN5500 may be useful even for the treatment of tumors exhibiting P-gp-mediated MDR.

Key words: KRN5500 — P-Glycoprotein — Multidrug resistance — Cellular pharmacokinetic analysis

The novel anticancer drug KRN5500 (6-[4-deoxy-4-(2*E*, 4*E*)-tetradecadienoylglycyl]amino-L-glycero-β-L-manno-heptopyranosyl]amino-9H-purine), developed in 1993, has a unique molecular structure not belonging to any category of anticancer drugs currently in clinical use (Fig. 1).[1,2] KRN5500 was demonstrated to be highly active in a number of experimental solid tumors, including stomach, colon and esophageal cancer.[3,4] KRN5500 exerts its growth-inhibitory effect after conversion to the active metabolite 4′-N-glycylspicamycin aminonucleoside (SAN-Gly) in the intracellular space of tumor cells.[3,5] SAN-Gly inhibits protein synthesis, a mechanism of action different from those of other anticancer drugs.[3,5,6] Currently, KRN5500 is under clinical investigation in Japan and the USA.[7]

Multidrug resistance (MDR) is one of the most serious problems responsible for the failure of chemotherapy. A well-characterized cellular phenotype of MDR is mediated by the multidrug transporter P-glycoprotein (P-gp), and P-gp is known to be frequently acquired in patients given chemotherapy.[8] P-gp, which is encoded by the *MDR1* gene, expels drugs from cells by utilizing the energy of ATP hydrolysis, and it has been found to be an efflux pump with broad substrate specificity.[9,10] It is important to determine whether anticancer drugs are substrates for P-gp, since substrates can be unexpectedly ineffective in such patients. The substrates for P-gp include vinblastine[11,12] and doxorubicin,[12] as well as the cardiac glycoside digoxin[13,14] and the immunosuppressive agent cyclosporin A.[15] It has been demonstrated *in vitro* that the cellular accumulation of vinblastine and doxorubicin is reduced[11,12] and they are not effective in P-gp-expressing MDR cells. Even for drugs that are substrates for P-gp, it should be clarified whether they are indeed transported by P-gp. Anticancer drugs that are not transported by P-gp will show little reduction in cellular accumulation in multidrug-resistant cells, and may therefore be effective in such cells. The MDR modulator PSC833,[12,16] Ca^{2+} antagonist nitrendipine[17] and the steroid hormone proge-

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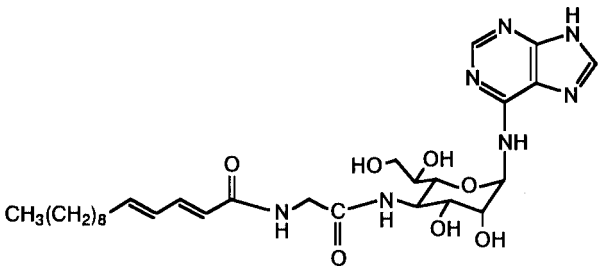

Fig. 1. Chemical structure of KRN5500.

sterone[18, 19] are known not to be transported by P-gp, resulting in comparable levels of cellular accumulation in multidrug-resistant and sensitive cells.

It has been demonstrated that the growth-inhibitory effect of KRN5500 in mouse leukemia P388 cells is comparable to that in doxorubicin-, vincristine- and mitomycin C-resistant P388 cells.[5] In contrast, Lee *et al*. reported that cisplatin-resistant human lung cancer cells showed higher sensitivity to KRN5500 as compared with sensitive cells.[20] Although these reports suggested that KRN5500 is not a substrate for P-gp, there is no direct evidence, and there is no information on the cellular kinetics of KRN5500, i.e., whether or not it is transported. Such information is necessary to determine appropriate dosage regimens of KRN5500.

Previously, we established the LLC-GA5-COL150 cell line, the cells of which express human P-gp on the apical membranes, by transfection of human *MDR1* cDNA into LLC-PK_1 cells.[13, 18] This cell line is a useful tool for identification of the substrates for P-gp and moreover for quantitative characterization of transport via P-gp.[11–15, 17, 18, 21, 22] Here, the interaction of KRN5500 with human P-gp was investigated using LLC-GA5-COL150 cells in addition to the parental cell line LLC-PK_1. [^{3}H]Azidopine photoaffinity labeling, cellular accumulation and transcellular transport were examined.

MATERIALS AND METHODS

Chemicals [^{14}C]KRN5500 (1.28 GBq/mmol) and non-labeled KRN5500 were kind gifts from Kirin Brewery Co., Ltd. (Tokyo). [^{3}H]Azidopine (1.81 TBq/mmol), [^{3}H]vinblastine (422 GBq/mmol), [^{3}H]inulin (25.2 GBq/mmol) and [methoxy-^{14}C]inulin (308 MBq/mmol) were obtained from Amersham International, plc (Buckinghamshire, UK). PSC833 was kindly supplied by Novartis Pharma (Basel, Switzerland). Colchicine, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and vinblastine sulfate were purchased from Wako Pure Chemical Industries, Ltd. (Osaka). All other chemicals were of the highest purity available.

Cells and cell culture LLC-GA5-COL150 cells were obtained by transfection of human *MDR1* cDNA into porcine kidney epithelial LLC-PK_1 cells, followed by culturing in the presence of 150 ng/ml colchicine.[13, 18] Cells were maintained in Medium199 (Dainippon Pharmaceutical Co., Ltd., Osaka) supplemented with 10% fetal bovine serum (BioWhittaker, Walkersville, MD) and 150 ng/ml of colchicine for LLC-GA5-COL150 cells without antibiotics.

Growth inhibition assay The growth-inhibitory effects of KRN5500 in LLC-GA5-COL150 and LLC-PK_1 cells were determined by colorimetric assay using MTT reagent.[18, 23, 24] Cells ($3–5\times10^3$ cells/well) were seeded on 96-well plates and cultured for 4 days in the absence or presence of KRN5500 at various concentrations. After culture, the medium was aspirated off and 50 μl of MTT solution (1 mg/ml) was added to each well. After incubation for 4 h, the supernatant was discarded, 100 μl of dimethyl sulfoxide was added to each well, and the absorbance was determined at 570 nm.

[^{3}H]Azidopine photoaffinity labeling of P-gp [^{3}H]Azidopine photoaffinity labeling of P-gp was performed as described previously.[18] The membrane fractions of the cells were obtained by sucrose gradient centrifugation after breaking up the cells by nitrogen cavitation (Mini-Bomb cell disruption chamber, Kontes Glass Co., Vineland, NJ). Membrane fractions (20 μg of protein) were reacted with [^{3}H]azidopine (0.4 μM, 37 kBq) in the presence or absence of the indicated concentration of KRN5500 or vinblastine at room temperature for 20 min. The reaction mixtures were then irradiated with a UV lamp (Black Ray Type XX-15L, Ultra-Violet Products, Upland, CA) for 30 min on ice. Photolabeled protein (20 μg) was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on 7.5% gels ("PAGEL," Atto Co., Tokyo). The gels were fixed, treated with fluorographic reagent "Amplify" (Amersham International), dried, and then exposed for 10 days at −80°C using "Hyperfilm" MP (Amersham International).

Cellular accumulation and transcellular transport of [^{14}C]KRN5500 Cellular accumulation and transcellular transport of [^{14}C]KRN5500 (1.4 μM, 3.7 kBq) and [^{3}H]vinblastine (100 nM, 37 kBq) were determined as described previously.[11–15, 17, 18, 21, 22] The transcellular transport assay system using LLC-GA5-COL150 and LLC-PK_1 cells is shown schematically in Fig. 2. The cells were seeded onto microporous polycarbonate membrane filters ("Transwell" 3414, Costar, Cambridge, MA) at confluent cell density and incubated for 3 days. The culture medium was replaced by fresh medium 3 h before the experiments. The medium on the basal or apical side of the monolayer was replaced with medium containing [^{14}C]KRN5500 and [^{3}H]vinblastine together with [^{3}H]inulin (0.73 μM, 37 kBq) and [methoxy-^{14}C]inulin (6 μM, 3.7 kBq), respectively,

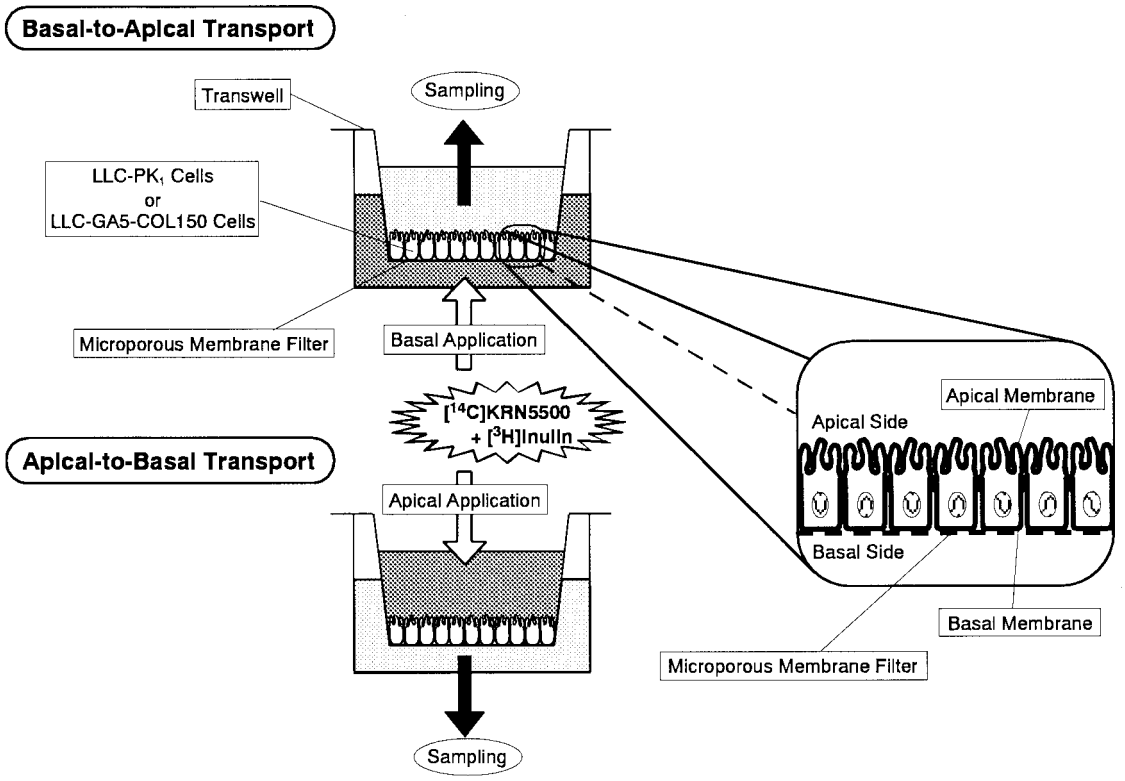

Fig. 2. Schematic illustration of the transcellular transport assay system using LLC-GA5-COL150 and LLC-PK_1 cells.

and aliquots of the medium on the opposite side were taken at the indicated times. To confirm the formation of tight junctions in the monolayers, paracellular leakage was monitored using radiolabeled inulin, which is a marker of paracellular transport. To examine cellular accumulation, immediately after the last sampling the cells were rapidly washed twice with ice-cold phosphate-buffered saline and lysed in 1 ml of 0.3 *N* NaOH. PSC833 (2 μM) was added to the medium on both sides of the cell monolayer 1 h before adding [^{14}C]KRN5500 or [^{3}H]vinblastine, and was also contained in the incubation medium throughout the experiments to examine its effects. The levels of radioactivity of the collected media and the lysed cells were counted in 3 ml of ACS II (Amersham International) by liquid scintillation counting (LSC-5100, Aloka Co., Ltd., Tokyo).

Protein assay Protein content was determined using a Bio-Rad Protein Assay Kit (Bio-Rad, Richmond, CA), with bovine γ-globulin as the standard.

Statistical analysis Statistical analysis of the data was performed by one-way analysis of variance followed by Scheffe's test. Statistical significance was defined as $P<0.05$.

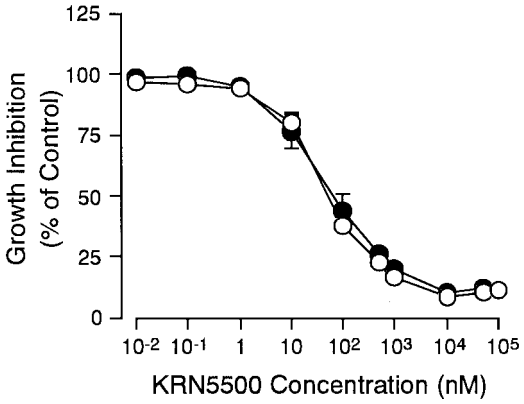

Fig. 3. Growth-inhibitory effect of KRN5500 in LLC-GA5-COL150 and LLC-PK_1 cells. Open circles (○) indicate LLC-PK_1 cells, and closed circles (●) indicate LLC-GA5-COL150 cells. Each point represents the mean±SE of three independent experiments.

RESULTS

Growth-inhibitory effect of KRN5500 The growth-inhibitory effect of KRN5500 in LLC-GA5-COL150 cells

was comparable to that in LLC-PK_1 cells (Fig. 3), with IC_{50} values of 79.4 and 72.7 n*M*, respectively, suggesting that KRN5500 was not transported by P-gp.

Inhibitory effect of KRN5500 on [^{3}H]azidopine photoaffinity labeling of P-gp To examine the interaction of KRN5500 with P-gp, the binding ability of KRN5500 for P-gp was examined in terms of the inhibition of [^{3}H]azidopine photoaffinity labeling of P-gp (Fig. 4). [^{3}H]Azidopine binding was observed only in the membrane fractions of LLC-GA5-COL150 cells, but not in LLC-PK_1 cells. Inhibition by KRN5500 was concentration-dependent; i.e., 40 μM KRN5500 showed partial, while 400 μM showed almost complete inhibition of [^{3}H]azidopine binding.

Cellular accumulation of [^{14}C]KRN5500 The cellular accumulation of [^{14}C]KRN5500 in LLC-GA5-COL150 and LLC-PK_1 cells was examined following its application on the basal side as well as the apical side (Table I). The cellular accumulation of [^{14}C]KRN5500 in LLC-GA5-COL150 cells was slightly lower than that in LLC-PK_1 cells after basal application, and co-administration of PSC833 restored it. For apical application, the cellular accumulation of [^{14}C]KRN5500 in LLC-GA5-COL150 cells was ca. 3-fold lower than that in LLC-PK_1 cells, and PSC833 had no effect on it.

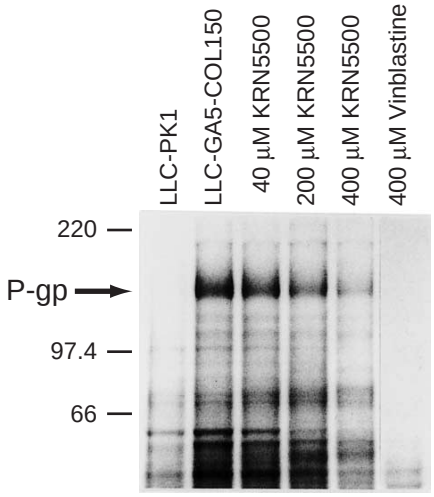

Fig. 4. Inhibitory effect of KRN5500 and vinblastine on [^{3}H]azidopine photoaffinity labeling of P-gp. Membrane fractions (20 μg of protein) from LLC-GA5-COL150 cells were photolabeled with [^{3}H]azidopine (0.4 μM) in the absence or presence of KRN5500 or vinblastine at the indicated concentrations. After solubilization, photolabeled protein was separated on SDS-PAGE. Molecular size standards are indicated in kilodaltons to the left.

Table I. Cellular Accumulation of [^{14}C]KRN5500 and [^{3}H]Vinblastine in LLC-GA5-COL150 and LLC-PK_1 Cells

Treatment	KRN5500 (pmol/mg protein/24 h)		Vinblastine (pmol/mg protein/3 h)	
	Basal apply	Apical apply	Basal apply	Apical apply
LLC-PK_1 cells	41.0±0.7	61.7±9.2	48.8±1.2	37.2±1.8
LLC-GA5-COL150 cells	27.8±1.0[b)]	19.8±1.4[b)]	2.8±0.7[b)]	0.5±0.1[b)]
+ 2 μM PSC833	41.0±2.1[c)]	13.9±0.8[b)]	28.8±5.4[a, c)]	25.0±6.2[c)]

a) and *b*) $P<0.05$ and $P<0.001$ significantly different from values of LLC-PK_1 cells with the same treatment, respectively.
c) $P<0.001$ significantly different from value of LLC-GA5-COL150 cells with the same treatment.
Values represent the mean±SE of four to eight independent experiments.

Table II. Transcellular Transport of [^{14}C]KRN5500 and [^{3}H]Vinblastine in LLC-GA5-COL150 and LLC-PK_1 Cells

Treatment	KRN5500 (% of dose at 24 h)		Vinblastine (% of dose at 3 h)	
	B-to-A[a)]	A-to-B	B-to-A	A-to-B
LLC-PK_1 cells	4.8±0.1	13.3±2.6	14.4±0.1	9.3±0.2
LLC-GA5-COL150 cells	6.8±0.2[c)]	5.4±0.1[c)]	21.6±1.3[b)]	1.9±0.3[c)]
+ 2 μM PSC833	5.3±0.1[e)]	5.0±0.2[b)]	11.6±1.6[d)]	7.0±0.9[b, e)]

a) B-to-A and A-to-B represent the basal-to-apical transport and the apical-to-basal transport, respectively.
b) and *c*) $P<0.05$ and $P<0.001$ significantly different from values of LLC-PK_1 cells with the same treatment, respectively.
d) and *e*) $P<0.01$ and $P<0.001$ significantly different from values of LLC-GA5-COL150 cells with the same treatment, respectively.
Values represent the mean±SE of four to eight independent experiments.

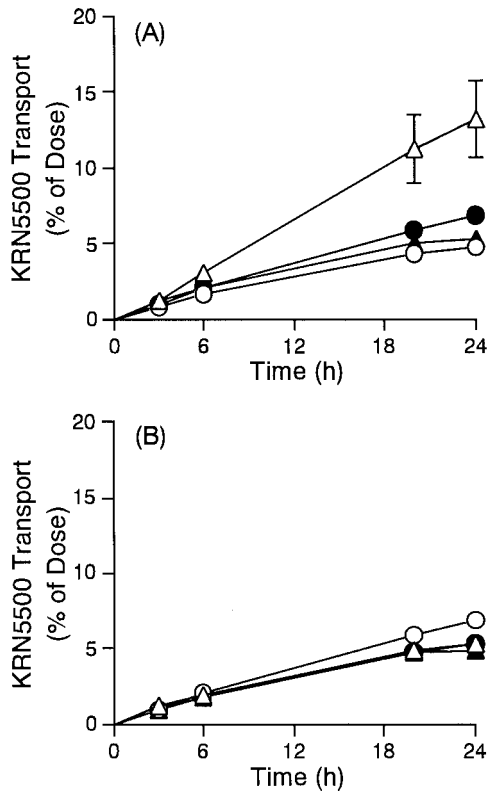

Fig. 5. Time course of transcellular transport of [^{14}C]KRN5500 in LLC-GA5-COL150 and LLC-PK$_1$ cells (A) and effect of PSC833 on transcellular transport of [^{14}C]KRN5500 in LLC-GA5-COL150 cells (B). A: The open symbols (○, △) indicate LLC-PK$_1$ cells, and closed symbols (●, ▲) show LLC-GA5-COL150 cells. B: The open symbols (○, △) indicate LLC-GA5-COL150 cells, and closed symbols (●, ▲) show LLC-GA5-COL150 cells in the presence of PSC833 (2 μM). The circles indicate the basal-to-apical transport and the triangles show the apical-to-basal transport. Each point represents the mean±SE of four to eight independent experiments.

Transcellular transport of [^{14}C]KRN5500 The transcellular transport of [^{14}C]KRN5500 after application on the basal side as well as the apical side of LLC-GA5-COL150 and LLC-PK$_1$ cell monolayers was also examined (Table II). The transcellular transport of [^{14}C]KRN5500 in LLC-GA5-COL150 cells as well as LLC-PK$_1$ cells was time-dependent with almost no alteration of the transport rate up to 24 h (Fig. 5). The basal-to-apical transport of [^{14}C]KRN5500 in LLC-GA5-COL150 cells was slightly higher than that in LLC-PK$_1$ cells. PSC833 had a little effect on the basal-to-apical transport of [^{14}C]KRN5500 in LLC-GA5-COL150 cells. However, the basal-to-apical transport of [^{14}C]KRN5500 in LLC-GA5-COL150 cells was only a little higher than the apical-to-basal transport, resulting in almost no directional transport of [^{14}C]-KRN5500 in LLC-GA5-COL150 cells. The apical-to-basal transport of [^{14}C]KRN5500 in LLC-PK$_1$ cells was markedly higher than the basal-to-apical transport, resulting in directional transport from the apical to the basal side.

DISCUSSION

To determine the optimal dosage regimen of anticancer drugs, it is helpful to clarify whether the anticancer drug is a substrate for P-gp, and moreover whether it is transported by P-gp. Here, the interaction of KRN5500 with P-gp was investigated using LLC-GA5-COL150 cells. The results for KRN5500 were compared with those for vinblastine, which is a substrate for and is transported by P-gp.[12)]

The growth-inhibitory effect of KRN5500 in LLC-GA5-COL150 cells was comparable to that in LLC-PK$_1$ cells, with IC$_{50}$ values of 79.4 and 72.7 n*M*, respectively, suggesting that KRN5500 was not transported by P-gp. This was different from the case of vinblastine, the IC$_{50}$ value of which in LLC-GA5-COL150 cells was about 90-fold higher than that in LLC-PK$_1$ cells (697 and 8 n*M*, respectively). However, KRN5500 inhibited the [^{3}H]azidopine binding in a concentration-dependent manner in the plasma membrane fraction derived from LLC-GA5-COL150 cells, although its binding affinity for P-gp was weaker than that of vinblastine, suggesting that KRN5500 was a substrate for P-gp. These results suggested that KRN5500 was a substrate for, but was not transported by P-gp.

Cellular accumulation and transcellular transport experiments were conducted to confirm the above findings. Following basal application, the cellular accumulation of [^{14}C]KRN5500 in LLC-GA5-COL150 cells was 1.5-fold lower than that in LLC-PK$_1$ cells (27.8 and 41.0 pmol/mg protein/24 h, respectively) and this was restored by PSC833 (2 μM). In contrast, that of [^{3}H]vinblastine in LLC-GA5-COL150 cells was nearly 20-fold lower than that in LLC-PK$_1$ cells (2.8 and 48.8 pmol/mg protein/3 h, respectively), and co-administration of PSC833 (2 μM) showed a restorative effect. The transcellular transport of [^{14}C]KRN5500 after its basal application in LLC-GA5-COL150 cells was also slightly higher than that in LLC-PK$_1$ cells, and the basal-to-apical transport of [^{14}C]KRN5500 was restored by 1.3-fold by PSC833 in LLC-GA5-COL150 cells. However, its basal-to-apical transport in LLC-GA5-COL150 cells was only a little higher than the apical-to-basal transport (6.8% and 5.4% of the dose at 24 h, respectively). In contrast, PSC833 markedly inhibited the transport of [^{3}H]vinblastine, and its basal-to-apical transport was about 20-fold higher than that in the opposite direction (21.6% and 1.9% of the dose at 3 h, respectively). These intrinsic cellular kinetics of KRN5500 were similar to those of PSC833,[12, 16)]

nitrendipine[17] and progesterone,[18, 19] all of which are substrates for, but are not transported by P-gp.

It was unexpected that the cellular accumulation of [^{14}C]KRN5500 after its apical application in LLC-GA5-COL150 cells was markedly lower (ca. 3-fold) than that in LLC-PK$_1$ cells. The apical-to-basal transport of [^{14}C]-KRN5500 in LLC-PK$_1$ cells was about 2.5-fold higher than that in LLC-GA5-COL150 cells, although the precise mechanism of this directional transport remains unclear. This could be explained by the increase in the incorporation of [^{14}C]KRN5500 into LLC-PK$_1$ cells due to its higher transport from the apical side.

Our results demonstrated that the novel anticancer drug KRN5500 interacted with, but was hardly transported by human P-gp. These results suggested that KRN5500 may be useful even for the treatment of tumor cells exhibiting P-gp-associated MDR.

ACKNOWLEDGMENTS

We thank Dr. Kazumitsu Ueda, Kyoto University Graduate School, for technical assistance with the LLC-GA5-COL150 cell culture. The authors are grateful to Kirin Brewery Co., Ltd. for providing the labeled and non-labeled KRN5500 and to Novartis Pharma for providing SDZ PSC833.

(Received July 22, 1999/Revised November 4, 1999/Accepted November 5, 1999)

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