Cellular Pharmacokinetic Aspects of Reversal Effect of Itraconazole on P-Glycoprotein-Mediated Resistance of Anticancer Drugs

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The reversal effect of itraconazole on P-glycoprotein (P-gp)-mediated resistance of vinblastine, daunorubicin and doxorubicin was analyzed from a cellular pharmacokinetic point of view, namely by [³H]azidopine photoaffinity labeling, intracellular accumulation and transcellular transport experiments. The LLC-GA5-COL150 cells, which expressed human P-gp selectively on the apical membrane due to transfection of *MDR1* cDNA into the porcine kidney epithelial cells (LLC-PK₁ cells), was used here, since this cell line constructs the monolayer with tight junction, being able to characterize the cellular pharmacokinetics. In LLC-GA5-COL150 cells, itraconazole caused a reversal from resistance as shown by a growth inhibition assay. [³H]Azidopine photoaffinity labeling demonstrated that itraconazole, vinblastine, daunorubicin and doxorubicin showed higher binding ability for P-gp compared with digoxin, suggesting the following results were *via* P-gp. The intracellular accumulation of [³H]vinblastine, [³H]daunorubicin and [¹⁴C]doxorubicin after their application on the basal and apical sides was increased by itraconazole. These changes were similar to the dose modifying factors determined by the growth inhibition assay. However, their basal-to-apical transport was hardly affected by itraconazole, and this was explained by the fact that itraconazole inhibited P-gp, and subsequently increased their intracellular concentration and then the non-P-gp mediated transport from the intracellular space to apical side. The apical-to-basal transport of [³H]vinblastine, [³H]daunorubicin and [¹⁴C]doxorubicin was increased by itraconazole, and this was reasonably explained by the inhibition of P-gp, and partly also by the increase of their intracellular concentration *via* the inhibition of P-gp.

Key words P-glycoprotein; multidrug resistance; reversal effect of itraconazole; cellular pharmacokinetic analysis

Multidrug resistance (MDR) remains a major obstacle in cancer chemotherapy. P-Glycoprotein (P-gp), which is encoded by the *MDR1* gene, actively transports a variety of structurally unrelated anticancer drugs out of cancer cells.²⁾ The resistance mechanism in MDR cells is mainly overexpression of P-gp on the plasma membrane. In contrast, MDR modulators, substances capable of blocking P-gp-mediated drug efflux, have been suggested to reverse P-gp-mediated drug resistance,³⁾ and finally to improve the outcome of cancer chemotherapy.^{4,5)}

Early MDR modulation clinical trials necessarily used the first generation MDR modulators, *i.e.*, drugs approved for other indications and had the advantage of easy availability as well as known toxicity profiles.⁵⁾ These include the antihypertensive verapamil, the immunosuppressive cyclosporin A, and so on. Later, S9788,⁶⁾ SDZ PSC 833 (PSC833)^{7,8)} and MS209⁹⁾ were developed as the second or third generation MDR modulators. Recently, it was reported that the antifungal agent, itraconazole also reversed daunorubicin resistance in P388/ADR cells, and adriamycin and etoposide resistance in human leukemia cells.^{10,11)} Itraconazole is understood to be one of the most useful MDR modulators from the view point of its frequent administration to patients treated with anticancer drugs.^{12,13)}

The reversal effects of these MDR modulators have been explained by the restoration of the intracellular accumulation of the anticancer drugs,^{6–11)} however, there was little information on their cellular pharmacokinetic aspects, which enables us to develop more effective and useful ones. Previously, we developed a system for evaluation of P-gp-mediated transcellular transport using human P-gp overexpressing

cells, LLC-GA5-COL150 cells.^{14,15)} This system was suitable for direct assessment of the amount that was actually pumped out of the cells *via* P-gp.^{14–21)} Herein, the cellular pharmacokinetic aspects of the reversal effect of itraconazole on P-gp-mediated resistance of anticancer drugs was assessed using LLC-GA5-COL150 cells.

MATERIALS AND METHODS

Chemicals Itraconazole was a gift from Janssen Pharmaceutical N.V. (Beerse, Belgium). PSC833 and cyclosporin A were kindly supplied by Novartis Pharma (Basel, Switzerland). [³H]Digoxin (595.7 GBq/mmol) and [³H]daunorubicin (185 GBq/mmol) were purchased from Du Pont-New England Nuclear (Boston, MA). [³H]Vinblastine (422 GBq/mmol), [¹⁴C]doxorubicin (2.11 GBq/mmol), [³H]inulin (25.2 GBq/mmol), [methoxy-¹⁴C]inulin (308 MBq/mmol) and [³H]-azidopine (1.81 TBq/mmol) were obtained from Amersham International, plc (Buckinghamshire, UK). Digoxin was purchased from Aldrich Chemical Co., Inc. (Milwaukee, WI). Vinblastine sulfate, daunorubicin hydrochloride, doxorubicin hydrochloride, colchicine and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Wako Pure Chemical Industries, Ltd. (Osaka). All other chemicals were of the highest purity available.

Cells and Cell Culture LLC-PK₁ and LLC-GA5-COL150 cells, which were established by transfection with human *MDR1* cDNA into LLC-PK₁ cells, were maintained by serial passage in plastic culture dishes (Iwaki Glass, Chiba).^{14–21)} Cells were incubated in complete medium consisting of Medium199 (Dainippon Pharmaceutical Co.,

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Ltd., Osaka) supplemented with 10% fetal bovine serum (BioWhittaker, Walkersville, MD) and 150 ng/ml of colchicine for LLC-GA5-COL150 cells without antibiotics. Monolayer cultures were grown in a humidified atmosphere of 5% CO₂-95% air at 37 °C, and were subcultured every 4 and 7 d for LLC-PK₁ and LLC-GA5-COL150 cells, respectively, with 0.02% EDTA and 0.05% trypsin (Gibco BRL, Life Technologies, Inc., Grand Island, NY).

Growth Inhibition Assay The sensitivities to anticancer drugs of LLC-PK₁ and LLC-GA5-COL150 cells were examined by a colorimetric assay using MTT reagent.^{15,22,23)} Cells (3–5 × 10³ cells/well) were seeded on 96-well plates, and cultured in a humidified atmosphere of 5% CO₂-95% air at 37 °C for 4 d in the absence or presence of anticancer drugs with or without itraconazole, cyclosporin A and PSC833. The concentration range of vinblastine, daunorubicin and doxorubicin was 10⁻²–10⁵ nM, and digoxin was not conducted at more than 10⁶ nM due to its non-cytotoxicity at this concentration. Itraconazole concentration was fixed at 0.5 μM, being around *C*_{max} values after its oral administration to human. The medium was aspirated off and 50 μl of MTT solution (1 mg/ml in phosphate-buffered saline) was added to each well. After incubation for 4 h, the supernatant was discarded and 100 μl of dimethyl sulfoxide was added to each well. The absorbance in each well was determined at 570 nm. The dose modifying factors of modulators were defined as the ratio between the IC₅₀ values in the absence and presence of modulators in LLC-GA5-COL150 cells.^{24,25)}

[³H]Azidopine Photoaffinity Labeling of P-gp [³H]-Azidopine photoaffinity labeling was performed as described previously.¹⁵⁾ Cells were broken in isotonic buffer by nitrogen cavitation (Mini-Bomb cell disruption chamber, Kontes Glass Co., Vineland, NJ), and the membrane fraction was obtained by sucrose gradient centrifugation. Plasma membranes (20 μg protein) were reacted with [³H]azidopine (0.4 μM, 37 kBq) in the absence and presence of test drugs at room temperature for 20 min. According to the previous reports, the concentration of test drugs was 1000-fold excess of azidopine as a probe, namely 400 μM.¹⁵⁾ The reaction mixture was then irradiated with UV lamp (Black Ray Type XX-15L, Ultra-Violet Products) for 30 min on ice. Photolabeled protein (20 μg) was loaded into each lane and separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on 7.5% gels (Pagel®, Atto, Co., Tokyo). The gels were treated with the fluorographic reagent Amplify™ (Amersham International) and exposed for 10 d at -80 °C using Hyperfilm™ MP (Amersham International).

Intracellular Accumulation and Transcellular Transport of Labeled Drugs Intracellular accumulation and transcellular transport of labeled drugs was determined as described previously.^{14–21)} Cells were seeded onto microporous polycarbonate membrane filters (3.0 μm pore size, inside diameter 24.5 mm; Transwell™ 3414, Costar, Cambridge, MA) at confluent cell density. Cells were cultured in a humidified atmosphere of 5% CO₂-95% air at 37 °C for 3 d. Three hours before the transport experiments, the culture medium was replaced with that without colchicine. In the case of digoxin, vinblastine or daunorubicin transport, medium on the donor side of the monolayers was replaced with 2 ml of fresh medium containing [³H]-labeled drug (100 nM, 37 kBq) together with [methoxy-¹⁴C]inulin (6 μM,

3.7 kBq). Medium on the receiver side was replaced with 2 ml of fresh medium alone. The monolayers were incubated in 5% CO₂-95% air at 37 °C, and aliquots (25 μl) of the incubation medium on the receiver side were taken at 1, 2 and 3 h. The paracellular leakage estimated by the amount of appearance of [methoxy-¹⁴C]inulin in the receiver side was less than 0.1% of the total radioactivity per hour. To examine the intracellular accumulation, immediately after the last sampling, cells were washed twice with ice-cold phosphate-buffered saline, and then solubilized in 1 ml of 0.3 N NaOH. In the case of doxorubicin transport, [¹⁴C]doxorubicin (800 nM, 3.7 kBq) together with [³H]inulin (734 nM, 37 kBq) was added to the donor side. As the transport rate of [¹⁴C]doxorubicin was slower than those of the other drugs, sampling was conducted at 3, 6, 20 and 24 h. The other procedures were the same as for the other drugs. To examine the effects of itraconazole on the intracellular accumulation and transcellular transport of labeled drugs, itraconazole was added to both sides of the monolayers 1 h before the experiments. The incubation medium also contained the same concentration of itraconazole throughout the transport experiments. Dimethyl sulfoxide (final concentration < 1%) used in dissolution of itraconazole did not affect the drug transport experiments. Radioactivities of the collected media and solubilized cells were counted in 3 ml of ACS II (Amersham International) by liquid scintillation counting (LS6000TA, Beckman Instruments, Inc., Fullerton, CA). Data are presented as percentages of the total radioactivity.

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 Scheffé's test. Statistical significance was defined as *p* < 0.05.

RESULTS

Effect of Itraconazole on P-gp-Mediated Resistance of Vinblastine, Daunorubicin and Doxorubicin The reversal effect of itraconazole on the P-gp-mediated resistance of vinblastine, daunorubicin and doxorubicin was evaluated by the growth inhibition assay using LLC-GA5-COL150 cells (Table 1). LLC-GA5-COL150 cells were highly resistant to these anticancer drugs compared with LLC-PK₁ cells. Itraconazole (0.5 μM) effectively reversed the resistance of vinblastine with the dose modifying factor of 11.4, followed by that of daunorubicin (1.2) and doxorubicin (2.1). The reversal effect of itraconazole was comparable to cyclosporin A, but was weaker than PSC833.

Inhibitory Effect of Itraconazole, Vinblastine, Daunorubicin and Doxorubicin on [³H]Azidopine Photoaffinity Labeling To elucidate the variation in the reversal effect of itraconazole on the resistance, the binding affinity of itraconazole, vinblastine, daunorubicin and doxorubicin for P-gp was examined by the [³H]azidopine photoaffinity labeling, where it was assessed by their inhibition of [³H]azidopine binding (Fig. 1). According to the previous reports, the concentration of test drugs was 1000-fold excess of azidopine as a probe, *i.e.*, 400 μM.¹⁵⁾ [³H]Azidopine binding was observed only in the membrane protein fraction from LLC-GA5-COL150 cells, but not LLC-PK₁ cells. Vinblastine, daunoru-

Table 1. Reversal Effects of Itraconazole, Cyclosporin A and PSC833 on P-gp-Mediated MDR

Treatment	IC ₅₀ (nM)			
	Digoxin	Vinblastine	Daunorubicin	Doxorubicin
LLC-PK ₁	10 ⁶ >	8±0.2	13±1.5	34±1.0
LLC-GA5-COL150	10 ⁶ >	697±6.8	2210±123	7040±713
+ 0.5 μM Itraconazole	10 ⁶ >	61±0.4 (11.4) ^{a)}	1810±249 (1.2)	3300±225 (2.1)
+ 0.5 μM Cyclosporin A	10 ⁶ >	559±7.8 (1.2)	917±15 (2.4)	2150±236 (3.3)
+ 0.5 μM PSC 833	10 ⁶ >	9±0.3 (76.6)	56±3.3 (39.8)	120±23 (58.7)

a) The values in the parenthesis represent the dose modifying factor of each modulator, which was defined as the ratio between the IC₅₀ values in the absence and presence of modulators. Values represent mean±S.E. of at least three independent experiments.

Fig. 1. Inhibitory Effects of Itraconazole, Digoxin, Vinblastine, Daunorubicin and Doxorubicin on [³H]Azidopine Photoaffinity Labeling of P-gp

Plasma membranes (20 μg protein) from LLC-PK₁ or LLC-GA5-COL150 cells were photolabeled with [³H]azidopine (0.4 μM) in the absence or presence of various drugs (400 μM). After solubilization, photolabeled protein was analyzed by SDS-PAGE. Lane 1 and 2: membrane protein alone from LLC-PK₁ and LLC-GA5-COL150 cells, respectively. Lanes 3, 4, 5, 6 and 7: membrane protein from LLC-GA5-COL150 cells in the presence of itraconazole, digoxin, vinblastine, daunorubicin, doxorubicin, respectively. Molecular size standards are indicated in kDa to the left.

bicin and doxorubicin inhibited the [³H]azidopine binding at the level of itraconazole, but at a higher level than digoxin.

Effect of Itraconazole on Intracellular Accumulation of [³H]Vinblastine, [³H]Daunorubicin and [¹⁴C]Doxorubicin
Intracellular accumulation of [³H]vinblastine, [³H]daunorubicin and [¹⁴C]doxorubicin following their application on the basal and apical sides of LLC-GA5-COL150 and LLC-PK₁ cells was also examined (Table 2). Their intracellular accumulation in LLC-GA5-COL150 cells was remarkably lower than in LLC-PK₁ cells, suggesting the overexpression of P-gp resulting in the secretion into extracellular space. Co-administration of itraconazole effectively restored the intracellular accumulation. The reversal ratio of [³H]vinblastine, [³H]daunorubicin and [¹⁴C]doxorubicin was 2.6–6.0 times, 1.6–2.3 times and 1.1–1.2 times, respectively, when applied on the basal side, and was 10.9–40.5 times, 2.4–4.1 times and 1.3–2.1 times, respectively, after the apical application. These were similar to the dose modifying factors (Table 1). A decrease of intracellular accumulation by overexpression of human P-gp and its restoration by itraconazole were also found for the apical application of [³H]digoxin. However, for its basal application, there was no difference between LLC-GA5-COL150 and LLC-PK₁ cells, and no effect of itraconazole on the intracellular accumulation in LLC-GA5-COL150 cells.

Effect of Itraconazole on Transcellular Transport of [³H]Vinblastine, [³H]Daunorubicin and [¹⁴C]Doxorubicin
Transcellular transport of [³H]vinblastine, [³H]daunorubicin and [¹⁴C]doxorubicin after their application on the basal side as well as the apical side of LLC-GA5-COL150 and LLC-PK₁ cells monolayer was also examined (Table 3). Basal-to-

apical transport of [³H]vinblastine, [³H]daunorubicin and [¹⁴C]doxorubicin was effectively increased, and simultaneously the apical-to-basal transport was significantly reduced by the overexpression of P-gp. Itraconazole restored only the apical-to-basal transport, and had hardly any effect on the basal-to-apical transport. For [³H]digoxin, the basal-to-apical transport across the LLC-GA5-COL150 cells monolayer was increased compared with LLC-PK₁ cells and the apical-to-basal transport was decreased in LLC-GA5-COL150 cells. Itraconazole decreased the basal-to-apical transport and increased the apical-to-basal transport in LLC-GA5-COL150 cells.

DISCUSSION

Itraconazole resulted in the reversal from the resistance by the growth inhibition assay in LLC-GA5-COL150 cells. The dose modifying factors of itraconazole for vinblastine, daunorubicin and doxorubicin were 11.4, 1.2 and 2.1, respectively. [³H]Azidopine photoaffinity labeling could not explain these dose modifying factors quantitatively, but qualitatively suggested the following kinetic experiments could be considered as P-gp relating phenomena. Intracellular accumulation of [³H]vinblastine, [³H]daunorubicin and [¹⁴C]doxorubicin after their application on the basal and apical sides of LLC-GA5-COL150 cells was remarkably lower than in LLC-PK₁ cells, and it was restored by itraconazole, corresponding to the dose modifying factors determined by the growth inhibition assay. Basal-to-apical transport of [³H]vinblastine, [³H]daunorubicin and [¹⁴C]doxorubicin was effectively increased, and simultaneously the apical-to-basal transport was significantly reduced by the overexpression of P-gp. Itraconazole restored only the apical-to-basal transport, and unexpectedly had hardly any effect on the basal-to-apical transport.

The experimental fact that there was only a slight effect of itraconazole on the basal-to-apical transport can be explained as follows: itraconazole inhibited the P-gp on the apical membrane, and subsequently increased the intracellular concentration of [³H]vinblastine, [³H]daunorubicin and [¹⁴C]doxorubicin, resulting in an increase of the non-P-gp mediated transport from the intracellular to out of the cells. Similarly, the fact that the apical-to-basal transport of [³H]vinblastine, [³H]daunorubicin and [¹⁴C]doxorubicin was increased by itraconazole, can be explained also by the increase of their transport according to an increase of intracel-

Table 2. Effects of Itraconazole on Intracellular Accumulation of [³H]Digoxin, [³H]Vinblastine, [³H]Daunorubicin and [¹⁴C]Doxorubicin in LLC-PK₁ and LLC-GA5-COL150 Cells

Treatment		Digoxin	Vinblastine	Daunorubicin	Doxorubicin
		pmol/mg protein/3 h			pmol/mg protein/24 h
LLC-PK ₁	Basal apply	2.3±0.29	11.1±0.94	26.2±1.37	49.3±1.22
	Apical apply	0.4±0.07	5.9±0.27	22.0±0.64	31.0±0.91
LLC-GA5-COL150	Basal apply	2.0±0.07	1.0±0.03	2.7±0.09	14.2±0.88
	Apical apply	0.2±0.02	0.1±0.00	1.2±0.05	4.9±0.07
+ 1 μM Itraconazole	Basal apply	2.1±0.10 (1.0) ^{a)}	2.6±0.01** (2.6)	4.2±0.16** (1.6)	15.1±0.80 (1.1)
	Apical apply	0.3±0.03* (1.8)	1.2±0.02** (10.9)	2.9±0.12** (2.4)	6.5±0.06 (1.3)
+ 100 μM Itraconazole	Basal apply	2.2±0.10 (1.1)	6.2±0.06** [†] (6.0)	6.2±0.15** [†] (2.3)	17.0±0.77 (1.2)
	Apical apply	0.4±0.05** (2.4)	4.5±0.03** [†] (40.5)	5.0±0.15** [†] (4.1)	10.3±0.88* (2.1)

a) The values in the parenthesis represent the ratio between the intracellular accumulation in the absence and presence of itraconazole. * and ** $p < 0.05$ and 0.001 significantly different from each value of LLC-GA5-COL150 cells treated with the same drug. [†] $p < 0.001$ significantly different from values of $1 \mu\text{M}$ itraconazole in the same drug treatment group. Values represent mean ± S.E. of at least three independent experiments.

Table 3. Effects of Itraconazole on Transcellular Transport of [³H]Digoxin, [³H]Vinblastine, [³H]Daunorubicin and [¹⁴C]Doxorubicin in LLC-PK₁ and LLC-GA5-COL150 Cells

Treatment		Digoxin	Vinblastine	Daunorubicin	Doxorubicin
		% of dose at 3 h			% of dose at 24 h
LLC-PK ₁	B-to-A ^{a)}	10.4±1.04	16.5±1.33	16.3±0.31	11.5±0.27
	A-to-B	4.4±0.33	3.5±0.26	16.5±0.25	8.2±0.27
LLC-GA5-COL150	B-to-A	16.9±0.88	22.3±0.46	34.0±0.99	34.3±0.45
	A-to-B	1.4±0.30	0.9±0.07	12.3±0.20	6.9±0.24
+ 1 μM Itraconazole	B-to-A	12.9±1.27 (0.8) ^{b)}	23.6±1.54 (1.1)	30.9±1.22 (0.9)	31.2±0.01 (0.9)
	A-to-B	3.9±0.30** (2.7)	2.9±0.12*** (3.1)	13.1±0.20 (1.1)	8.2±0.34 (1.2)
+ 100 μM Itraconazole	B-to-A	9.3±0.89** (0.6)	22.0±0.61 (1.0)	30.1±0.76* (0.9)	28.2±0.99** (0.8)
	A-to-B	6.8±0.54*** [†] (4.8)	6.0±0.43*** ^{††} (6.4)	13.6±0.58 (1.1)	8.0±0.47 (1.2)

a) B-to-A and A-to-B represent the basal-to-apical transport and the apical-to-basal transport, respectively. b) The values in the parenthesis represent the ratio between the transport in the absence and presence of itraconazole. *, ** and *** $p < 0.05$, 0.01 and 0.001 significantly different from each value of LLC-GA5-COL150 cells treated with the same drugs, respectively. [†] and ^{††} $p < 0.01$ and 0.001 significantly different from values of $1 \mu\text{M}$ itraconazole in the same drug treatment group, respectively. Values represent mean ± S.E. of at least three independent experiments.

lular concentration *via* the inhibition of P-gp on the apical membrane in addition to the decrease of back flux *via* P-gp. These results would allow us to understand the reversal effect of MDR modulators more exactly.

For [³H]digoxin, the basal-to-apical transport across the LLC-GA5-COL150 cells monolayer was increased compared with LLC-PK₁ cells and the apical-to-basal transport was decreased in LLC-GA5-COL150 cells. Itraconazole inhibited the basal-to-apical transport and increased the apical-to-basal transport in LLC-GA5-COL150 cells. The intracellular accumulation after the apical application onto LLC-GA5-COL150 cells was lower than in LLC-PK₁ cells, and was restored by itraconazole; however, this phenomena was not found after the basal application. This can be partly explained by the human P-gp being located only in the apical membrane. The reversal ratios of [³H]vinblastine, [³H]daunorubicin and [¹⁴C]doxorubicin were larger after the apical application than after the basal application, and this was also caused by the localization of human P-gp.

In this series of experiments, LLC-GA5-COL150 cells were usually compared with LLC-PK₁ cells,^{14–21)} and there

was no data on the cells with transfection of empty vector into LLC-PK₁ cells. The transfection process could affect the overall results and the experimental fact that itraconazole increased the apical-to-basal transport of [³H]digoxin and [³H]vinblastine in LLC-GA5-COL150 cells more than that in LLC-PK₁ cells can be derived from this factor.

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