Reversal Effects of Antifungal Drugs on Multidrug Resistance in MDR1-Overexpressing HeLa Cells

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In this study, the antiproliferative effects of vinblastine (VLB), paclitaxel (TXL), doxorubicin (DXR), daunorubicin (DNR) and 5-fluorouracil (5-FU) were assessed in the human cervical carcinoma cell line HeLa-Ohio (HeLa) and Hvr100-6 cells, established by growing the parental HeLa cells in the presence of progressively greater concentrations of VLB in the culture medium. Flow cytometric analysis indicated the induction of MDR1 (P-glycoprotein) in Hvr100-6 cells with no alterations in levels of multidrug resistance-associated protein (MRP). Resistance to VLB, TXL, DXR and DNR was found in Hvr100-6 cells with relative resistances of *ca.* 300, 4000, 50 and 200, respectively, whereas no resistance was found to 5-FU. The reversal effects of antifungal drugs, fluconazole, itraconazole, ketoconazole, miconazole and amphotericin B on multidrug resistance were also assessed using Hvr100-6 cells. Itraconazole was found to have potent reversal effect on the resistance to VLB and TXL, but the others had no such effect. This reversal effect of itraconazole was concentration-dependent, with dose modifying factors of 3.2, 10.1 and 435.7 at 0.1, 0.25 and 0.5 μM of itraconazole, respectively. In addition, this reversal effect of itraconazole was explained by the inhibition of accumulation of the anticancer drugs.

Key words MDR1; itraconazole; reversal of MDR

Until the introduction of the azole antifungal drugs ketoconazole in 1981, fluconazole in 1990 and itraconazole in 1992, the treatment of most invasive fungal infections was essentially limited to intravenous injection of toxic amphotericin B with or without 5-flucytosine.^{1–3)} The availability of orally absorbed azoles has enabled outpatient therapy of deep mycoses, including Aspergillosis and Cryptococcosis, and long-term prophylaxis of immunocompromised patients with HIV infection or severe tumors. However, with growing usage of azoles, the clinical significance of pharmacokinetic drug–drug interactions has become increasingly clear. *In vitro* investigations using human liver microsomes have indicated that the azole antifungal drugs are all inhibitors of cytochrome P450 (CYP) enzymes, but their potencies are different for different isoforms.^{1–3)} Human CYP3A4, the major adult isoform, is expressed in the liver and small intestine and is inhibited by ketoconazole, and less strongly by itraconazole and fluconazole^{3,4)}; this is understood to account for most of the clinical drug interaction.

Recently, it has been postulated that there is considerable overlap in substrate specificity of CYP3A4 and multidrug transporter MDR1 (P-glycoprotein),^{5–9)} which was originally isolated from multidrug-resistant tumor cells and is expressed in normal tissues, and is also responsible for drug interactions.^{10,11)} However, the data on the interactions of antifungal drugs with MDR1 come from *in vitro* cell models or *mdr1a*(–/–) knock-out mice, making definitive conclusions difficult.³⁾ Similarly to CYP3A4, the information on the rank order of azole antifungal drugs from the viewpoint of interaction with MDR1 will be useful when selecting one of these drugs for patients taking drugs that are MDR1 substrates or for those bearing tumors and undergoing chemotherapy.

In this study, the vinblastine (VLB)-resistant HeLa derivative cell line, Hvr100-6, was established by stepwise ex-

posure to VLB, and MDR1 expression was analyzed by flow cytometry. Antiproliferative effects of VLB, paclitaxel (TXL), doxorubicin (DXR), daunorubicin (DNR) and 5-fluorouracil (5-FU) were assessed using HeLa and Hvr100-6 cells, and the antifungal drugs fluconazole, itraconazole, ketoconazole, miconazole and amphotericin B were compared from the viewpoint of the reversal effect. In addition, the mechanism underlying the alteration of cytotoxicity was examined by accumulation analysis using [³H]VLB.

MATERIALS AND METHODS

Chemicals Fluconazole was a kind gift from Pfizer Pharmaceuticals Inc. (Tokyo, Japan), itraconazole was from Janssen Pharmaceutical N.V. (Beerse, Belgium), and miconazole was from Mochida Pharmaceutical Co., Ltd. (Tokyo, Japan). Ketoconazole was purchased from ICN Biochemicals Inc. (Aurora, OH, U.S.A.). Amphotericin B, vinblastine sulfate (VLB), paclitaxel (TXL), doxorubicin hydrochloride (DXR), daunorubicin hydrochloride (DNR), and 5-fluorouracil (5-FU) were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). [³H]VLB (422 GBq/mmol) was obtained from Amersham International, plc (Buckinghamshire, U.K.). Other chemicals were of the highest purity available.

Culture of HeLa and Hvr100-6 Cells The human cervical carcinoma cell line HeLa-Ohio (HeLa) was obtained from Dainippon Pharmaceutical Co., Ltd. (Osaka, Japan). HeLa cells (396–401 passage) were maintained in culture medium consisting of Dulbecco's modified Eagle's medium (D-MEM with glucose (4.5 g/l), L-glutamine (4 mM) and sodium pyruvate (1 mM); Cat. No. 12800-017, Gibco BRL, Life Technologies, Inc., NY, U.S.A.) supplemented with 10% fetal bovine serum (FBS; Lot. No. AGM7413, HyClone, UT,

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U.S.A.) and 100 mg/l kanamycin sulfate (Gibco BRL). A VLB-resistant subline of HeLa, Hvr100-6, was established by stepwise increases of VLB concentration in the culture medium (5×10^6 cells/60 mm dish). Hvr100-6 cells (82–87 passage) were derived and maintained with 100 nM VLB. Both of these cell lines ($4\text{--}12 \times 10^4$ cells/cm²) were seeded into culture flasks (NuncTM flasks, Nalge Nunc International, U.S.A.), grown in a humidified atmosphere of 5% CO₂–95% air at 37 °C, and subcultured every 3 or 4 d with 0.05% trypsin–0.02% EDTA (Gibco BRL).

Flow Cytometric Analysis of MDR1 and MRP in HeLa and Hvr100-6 Cells HeLa and Hvr100-6 cells were treated with EDTA and harvested in Dulbecco's modified phosphate-buffered saline without magnesium and calcium (PBS; 137.9 mM NaCl, 8.1 mM Na₂HPO₄, 2.7 mM KCl, 1.5 mM KH₂PO₄). Both cell lines (1×10^6 cells) were incubated with the MDR1-specific monoclonal antibodies MRK-16 (1 : 5 dilution, Funakoshi Co., Ltd., Tokyo, Japan)¹² and JSB-1 (1 : 10 dilution, Sanbio BV, Netherlands),¹³ or the multidrug resistance-associated protein (MRP)-specific monoclonal antibody MRPM6 (1 : 20 dilution, Sanbio BV)¹⁴ for 60 min on ice. As a secondary antibody, FITC-conjugated goat anti-mouse IgG (Becton Dickinson & Co., NJ, U.S.A.) was used, and the cells were incubated for 30 min on ice. The cells were washed twice with PBS, and the fluorescence intensity of aliquots of 1×10^5 cells was determined using a fluorescence-activated cell sorter (FACScanTM, Becton Dickinson & Co.).

Antiproliferative Effects of Anticancer Drugs in HeLa and Hvr100-6 Cells Antiproliferative effects of VLB, TXL, DXR, DNR, and 5-FU were assessed in HeLa and Hvr100-6 cells by the WST-1 (tetrazolium salts) colorimetric assay using a Cell Counting Kit (Dojindo Laboratories, Kumamoto, Japan). Cells (1000 cells/well) were seeded on 96-well plates (Nalge Nunc International) in 100 μ l of culture medium without any anticancer drug on Day 0, and the culture medium was exchanged to that containing test anticancer drug at various concentrations on Day 1. The anticancer drugs were added using ethanol, dimethyl sulfoxide or double distilled water. The maximum concentration of organic solvents or double distilled water was 1%, which had no effect on cell viability or growth. After incubation for 3 d at 37 °C (on Day 4), the culture medium was exchanged to 110 μ l of that containing WST-1 reagent solution (10 μ l WST-1 solution and 100 μ l the culture medium), and 3 h later, the absorbance was determined at 450 nm with a reference wavelength of 630 nm using a microplate reader (Sjeia Auto Reader II; Sanko Junyaku Co., Ltd., Tokyo, Japan) according to the Manufacturer's directions. Preliminary experiments demonstrated a good proportional relationship between the absorbance and cell number (ranging from 500 to 32000 cells/well) with acceptable precision and accuracy and very small day-to-day or within-day variations.

The 50% growth inhibitory concentration (IC₅₀) of the anticancer drugs in HeLa and Hvr100-6 cells was calculated according to the sigmoid inhibitory effect model as follows using the nonlinear least-squares fitting method (WinNonlin[®], ver. 2.1, Pharsight Corp., CA, U.S.A.):

$$E = E_{\max} \times [1 - C^\gamma / (C^\gamma + IC_{50}^\gamma)]$$

where E and $E_{\max}$ represent the surviving fraction (% of control) and its maximum, respectively, and C and γ represent

the drug concentration in the medium (pM, nM or μ M) and the sigmoidicity factor, respectively. Each series of experiments was conducted in quadruplicate, and values are presented here as means and S.D. after rounding off to three significant figures. The relative resistance to an anticancer drug of Hvr100-6 cells compared with HeLa cells was defined as the ratio of IC₅₀ values.

Reversal Effects of Antifungal Drugs on Multidrug Resistance The reversal effects of antifungal drugs, fluconazole, itraconazole, ketoconazole, miconazole, and amphotericin B, on the multidrug resistance were also assessed by the WST-1 colorimetric assay using a Cell Counting Kit. An antifungal drug was added simultaneously with an anticancer drug on Day 1. The dose modifying factor (DMF) of an antifungal drug was defined as the ratio of IC₅₀ values in Hvr100-6 cells in the absence and presence of antifungal drug.^{15,16}

[³H]VLB Accumulation Study in HeLa and Hvr100-6 Cells Cells (2×10^5 cells/ml/well) were seeded on 24-well plates (Nalge Nunc International) in culture medium without VLB, and cultured for 2 d in a humidified atmosphere of 5% CO₂–95% air at 37 °C. The cells were washed twice with warmed complete Hank's balanced salt solution (HBSS, Cat. No. H-6136, Sigma Chemical), followed by preincubation with HBSS containing itraconazole (0.1, 0.5, 1, 10 and 100 μ M) for 1 h. The accumulation studies were immediately started by replacement with HBSS containing itraconazole and [³H]VLB (1 μ M, 18.5 kBq/ml/well). In the control experiments, itraconazole was not included for preincubation or incubation. High concentration of [³H]VLB was necessary for accurate determination of cellular radioactivity. Two hours later, the experiment was terminated by aspiration of HBSS from each well. Preliminary experiments had demonstrated that [³H]VLB content in the cells attained at steady-state by the incubation for 90 min (data were not shown). The cells were then quickly washed three times with ice-cold PBS on ice, and subsequently lysed in 1 ml of 0.3 N NaOH. It was confirmed that there was no detectable radioactivity in the washing ice-cold PBS. Radioactivity of the lysed cells was counted in 3 ml of ACS II (Amersham International) by liquid scintillation counting (LS6000TA, Beckman Instruments, Inc., CA, U.S.A.). The radioactivity in the washing solution was also determined in each experiment, and was usually around the level of background.

Protein Assay Protein content was determined using a Bio-Rad Protein Assay kit (Bio-Rad Laboratories, CA, U.S.A.). Bovine γ -globulin was used as the standard.

Statistical Analysis Multiple comparisons were performed by one-way analysis of variance (ANOVA) followed by Scheffe's test. p values of less than 0.05 (two-tailed) were considered significant.

RESULTS

Flow Cytometric Analysis of MDR1 and MRP in HeLa and Hvr100-6 Cells Figure 1 shows the fluorescence histograms for HeLa and Hvr100-6 cells, in which the results on three antibodies were superimposed with those from treatment with only the second antibody. Flow cytometric analysis with MRK-16 and MRPM6 indicated the induction of MDR1 by stepwise exposure to VLB with no induction of

Fig. 1. Fluorescence Histograms for HeLa (Left) and Hvr100-6 Cells (Right) Obtained by Flow Cytometric Analysis

MRK-16 and JSB-1 were the MDR1-specific monoclonal antibodies recognizing the extracellular and intracellular epitopes of MDR1, respectively. MRPm6 was the multidrug resistance-associated protein (MRP)-specific monoclonal antibody. The signals obtained with the cells only, and treatment only with the 2nd antibody are also superimposed.

Fig. 2. Growth Inhibitory Curves of HeLa and Hvr100-6 Cells by Paclitaxel and 5-Fluorouracil

Open and closed circles represent the growth inhibitory curves of HeLa and Hvr100-6 cells in the absence of itraconazole, respectively. Closed triangles represent those of Hvr100-6 cells in the presence of 0.5 μM itraconazole. Each point represents the mean $\pm$ S.D. of at least four experiments.

Table 1. Effects of Antifungal Drugs on the Cytotoxicity of Anticancer Drugs in HeLa and Hvr100-6 Cells

	Vinblastine IC ₅₀ (nM)	Paclitaxel IC ₅₀ (nM)	Doxorubicin IC ₅₀ (nM)	Daunorubicin IC ₅₀ (nM)	5-Fluorouracil IC ₅₀ (μM)
HeLa Cells	0.135 $\pm$ 0.036 ^{a)}	0.136 $\pm$ 0.238	2.94 $\pm$ 0.535	1.09 $\pm$ 0.309	2.85 $\pm$ 0.747
Hvr100-6 Cells	67.0 $\pm$ 30.1	562 $\pm$ 255	153 $\pm$ 56.8	216 $\pm$ 87.3	11.5 $\pm$ 2.33
Triazoles					
+0.1 μM Fluconazole	39.1 $\pm$ 46.9	469 $\pm$ 188	558 $\pm$ 579	191 $\pm$ 114	21.2 $\pm$ 14.7
+0.1 μM Itraconazole	30.4 $\pm$ 11.0	175 $\pm$ 351	528 $\pm$ 451	204 $\pm$ 137	25.3 $\pm$ 17.1
Imidazoles					
+0.1 μM Ketoconazole	88.1 $\pm$ 30.6	474 $\pm$ 279	477 $\pm$ 391	157 $\pm$ 74.5	7.15 $\pm$ 3.65
+0.1 μM Miconazole	51.7 $\pm$ 46.3	394 $\pm$ 107	212 $\pm$ 256	221 $\pm$ 180	4.39 $\pm$ 5.43
Polyenemacrolide					
+0.1 μM Amphotericin B	86.9 $\pm$ 71.3	633 $\pm$ 385	326 $\pm$ 146	95.7 $\pm$ 145	11.3 $\pm$ 7.51

a) The values are the means $\pm$ S.D. of four independent experiments.

MRP. No shift in signal obtained was found with JSB-1 antibody, which was shown to recognize the intracellular epitope of MDR1, suggesting that there was no abnormal orientation of the expression of MDR1.

Antiproliferative Effects of Anticancer Drugs in HeLa and Hvr100-6 Cells and Reversal Effects of Antifungal Drugs Figure 2 shows the growth inhibitory curves of HeLa and Hvr100-6 cells by TXL and 5-FU, a well-documented substrate and non-substrate of MDR1, respectively. The growth curves of Hvr100-6 cells in the presence of 0.5 μM itraconazole are also indicated. The growth curve for

TXL was shifted to a higher concentration in Hvr100-6 cells as compared with HeLa cells, with relative resistance of 4145. For 5-FU, the growth curve of Hvr100-6 cells was comparable to that of HeLa cells. Itraconazole (0.5 μM) reversed the resistance to TXL in Hvr100-6 cells, whereas it had no effect on 5-FU cytotoxicity.

Table 1 summarizes the antiproliferative effects of anticancer drugs in HeLa and Hvr100-6 cells and their alterations by antifungal drugs at 0.1 μM. Resistance to VLB, TXL, DXR and DNR was found in Hvr100-6 cells with relative resistances of *ca.* 300, 4000, 50 and 200, respectively,

Table 2. Concentration-Dependent Effects of Itraconazole on the Cytotoxicity of Paclitaxel in Hvr100-6 Cells

	IC ₅₀ (nM)	RR ^{b)}	DMF ^{c)}
HeLa Cells	0.136±0.238 ^{a)}	—	—
Hvr100-6 Cells	562±255	4145	—
+0.1 μM Itraconazole	175±351	1287	3.2
+0.25 μM Itraconazole	55.7±21.6	410	10.1
+0.5 μM Itraconazole	1.29±0.583	9.5	435.7

a) The values are the means±S.D. of four independent experiments. b) RR: relative resistance, the ratio of IC₅₀ value for Hvr100-6 cells to that for HeLa cells. c) DMF: dose modifying factor, the ratio of IC₅₀ value for Hvr100-6 cells in the absence to that in the presence of itraconazole.

Table 3. Cytotoxicity of Antifungal Drugs in HeLa and Hvr100-6 Cells

	IC ₅₀ (μM)		RR ^{b)}
	HeLa	Hvr100-6	
Triazole derivatives			
Fluconazole	137±132 ^{a)}	228±98.9	1.66
Itraconazole	5.35±4.78	2.14±0.726	0.40
Imidazole derivatives			
Ketoconazole	28.1±16.6	18.9±11.4	0.673
Miconazole	14.8±5.47	11.6±4.39	0.784
Polyenemacrolide			
Amphotericin B	3.29±0.764	4.38±0.669	1.33

a) The values are the means±S.D. of four independent experiments. b) RR: relative resistance, the ratio of IC₅₀ value for Hvr100-6 cells to that for HeLa cells.

whereas no resistance was seen to 5-FU. None of the antifungal drugs used here had any effects on the antiproliferative effects of any of the anticancer drugs examined, except that itraconazole was suggested to restore the resistance to VLB and TXL in Hvr100-6 cells. This reversal effect of itraconazole was concentration-dependent, with DMF values of 3.2, 10.1 and 435.7 at 0.1, 0.25 and 0.5 μM of itraconazole, respectively (Table 2).

Table 3 indicates the antiproliferative effects of antifungal drugs in HeLa and Hvr100-6 cells. There were no differences in the IC₅₀ values between HeLa and Hvr100-6 cells. The value of IC₅₀ for itraconazole was more than 10-fold higher than that showing the reversal of resistance.

[³H]VLB Accumulation Study in HeLa and Hvr100-6 Cells The [³H]VLB accumulation study was conducted to confirm the mechanism underlying the alterations in cytotoxicity. Accumulation amount of [³H]VLB in Hvr100-6 cells was about 10-fold lower than that in HeLa cells. Itraconazole had no effect on [³H]VLB accumulation in HeLa cells, but increased the [³H]VLB accumulation in Hvr100-6 cells, consistent with the data regarding cytotoxicity.

DISCUSSION

Synergistic action of anticancer drugs and antifungal drugs has been discussed in relation to the alteration of membrane lipid composition caused by antifungal drugs.^{17–19)} The alteration of antiproliferative effects of antifungal drugs following virus-transformation has also been explained, in part, by the increase of penetration through membrane barrier.^{17–19)} In this study, multidrug resistant Hvr100-6 cells were established by stepwise exposure to VLB, and resistance to antifungal drugs was not found in Hvr100-6 cells with relative

Fig. 3. Accumulation of [³H]Vinblastine by HeLa and Hvr100-6 Cells in the Absence or Presence of Itraconazole

Cells were incubated in Hank's balanced salt solution containing 1 μM [³H]VLB (37 kBq) for 2 h at 37 °C. Open and closed columns indicate the accumulation in the absence and presence of itraconazole, respectively. Each bar represents the mean±S.D. of quadruplicate experiments.

resistance of around unity (Table 3). None of the antifungal drugs used here had any effects on the antiproliferative effects of any of the anticancer drugs examined, except that itraconazole was suggested to restore the resistance to VLB and TXL in Hvr100-6 cells. (Table 1). Effect of itraconazole was concentration-dependent (Table 2), and was explained by alteration of accumulation of anticancer drugs (Fig. 3). Collectively, the reversal effect of itraconazole on resistance to VLB and TXL was expected to be *via* MDR1 rather than alteration of membrane permeability. Unexpectedly, itraconazole had no such effect on the resistance to DXR and DNR in Hvr100-6 cells, which were well-documented substrates of MDR1. This discrepancies might be due to much expression of MDR1 in the cells, that is, 0.1 μM of itraconazole was not enough to inhibit MDR1 function in the case of DXR and DNR, but further evaluation should be made.

In vitro experiments using human liver microsomes suggested that ketoconazole is a highly potent inhibitor of CYP3A4, followed by itraconazole and fluconazole. Since itraconazole shows a higher intrahepatic concentration than inhibition constant, clinically significant drug interactions were found for itraconazole.³⁾ In contrast, insufficient data are available regarding the interaction of antifungal drugs with MDR1, although MDR1 could be responsible for drug interactions as well as CYPs and it has been postulated that there is considerable overlap in substrate specificity of CYP3A4 and multidrug transporter MDR1.^{5–9)} Ketoconazole completely inhibited the MDR1-mediated transport of [³H]digoxin across Caco-2 monolayers at 10 μM, and the data obtained in knock-out mice suggested that it was a substrate of MDR1.⁵⁾ At a concentration of 10 μg/ml, ketoconazole reversed vinblastine and doxorubicin resistance in KB-V1 cells derived from human KB carcinoma cells.²⁰⁾ In contrast, ketoconazole shows only slight inhibition of the transport of rhodamine 123 across Caco-2 monolayers even at 100 μM.²¹⁾ Itraconazole also inhibited the MDR1-mediated transport of [³H]digoxin across monolayers of MDR1-overexpressing LLC-GA5-COL150 cells at 1 μM.²²⁾ Reversal of resistance has also been reported for itraconazole, where daunorubicin and adriamycin resistance was reversed at a concentration of 1–2.5 μg/ml in P388/ADR²³⁾ and K562/ADR,²⁴⁾ respectively. The data on the interactions of antifungal drugs with MDR1 varied depending on the experimental system, and no

information is available regarding the rank order of antifungal drugs from the viewpoint of interactions with MDR1. In this study, only itraconazole suppressed the function of MDR1 at achievable plasma concentrations in humans, suggesting that the pharmacokinetic drug-drug interactions via MDR1 might occur or the resistance might be reversed when itraconazole is administered as an antifungal drugs.

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