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Effect of micafungin on cytochrome P450 3A4 and multidrug resistance protein 1 activities, and its comparison with azole antifungal drugs

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Abstract

The effects of micafungin on cytochrome P450 3A4 (CYP3A4) metabolic and multidrug resistance protein 1 (MDR1) transport activities were investigated and compared with those of amphotericin B and four azole antifungal drugs (ketoconazole, itraconazole, fluconazole and miconazole). The effects on the metabolic activity of CYP3A4 were examined by measuring nifedipine oxidase activity in human liver microsomes and the effects on MDR1 transport activity were evaluated using [^{3}H]digoxin in MDR1-overexpressing LLC-GA5-COL150 cells. An inhibitory effect on CYP3A4 activity was found for ketoconazole, itraconazole and miconazole, with 50% inhibitory concentrations of 11.7, 32.6 and 74.2 nM, respectively. Fluconazole and micafungin had only slight inhibitory effects and amphotericin B had no effect. The MDR1-mediated transport of [^{3}H]digoxin was inhibited by ketoconazole and itraconazole, and slightly by miconazole. It is suggested that micafungin and amphotericin B would be unlikely to cause drug–drug interactions by inhibition of CYP3A4 and MDR1. A positive correlation between the inhibitory effects on CYP3A4 and MDR1 activities was observed, and the physicochemical mechanisms involved and impact on clinical treatment should be studied further.

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Introduction

Until the introduction of azole antifungal drugs during the 1980s and 1990s, most invasive fungal infections were treated almost exclusively by intravenous injection of amphotericin B with or without 5-flucytosine (Venkatakrishnan et al 2000; Denning 2003; Jarvis et al 2004). The development of orally absorbed azoles has enabled outpatient therapy of deep mycoses, including aspergillosis and cryptococcosis, and long-term treatment of immunocompromized patients with HIV infection or severe tumours. The target of azoles is sterol 14-α-demethylase, a microsomal cytochrome P450 (CYP), Ergo11p or CYP51p-dependent enzyme system, resulting in an impairment of biosynthesis of ergosterol for the cytoplasmic membrane (Odds et al 2003), and therefore the clinical significance of pharmacokinetic drug interactions has become increasingly clear with the growing usage of azoles. In-vitro investigations using human liver microsomes have confirmed that the azoles are all inhibitors of CYP enzymes and that most of the drug interaction is explained by the effects on the metabolic activity of CYP3A4, which is the major adult isoform expressed in the liver and small intestine. In the late 1990s, the echinocandin antifungal drugs, including micafungin, caspofungin and anidulafungin, were developed, targeting the synthetic cell-wall enzyme complex β-1,3-D-glucan synthase. Fungi are eukaryotes like humans, but the cell wall is not shared, and therefore adverse events involving echinocandins are mild and little CYP-mediated drug interaction is expected to occur. The inhibitory effects of azoles and echinocandin on CYP3A4 should be compared in terms of drug–drug interactions.

CYP3A4 and multidrug resistance protein 1 (MDR1) (P-glycoprotein) have been found to show significant overlap in substrate or inhibitor specificity, and it has been proposed that MDR1 would regulate the access of drugs to CYP3A4 in the intestine

(Wacher et al 1995, 1998, 2001; Wandel et al 1999; Benet et al 2003). MDR1 is now recognized to be an important protein that regulates the pharmacokinetics of various types of structurally unrelated drugs by transporting them from the inside to the outside of the cell (Lin & Yamazaki 2003; Sakaeda et al 2003; Schinkel and Jonker 2003; Schwab et al 2003; Marzolini et al 2004). In addition, MDR1 has recently been found to be a key in the interaction of digoxin with calcium antagonists (Takara et al 2002a, b) or lovastatin and simvastatin (Sakaeda et al 2002), but little information is available regarding MDR1-mediated drug interactions compared with CYPs.

In the present study, the effect of micafungin, a recently developed novel echinocandin, on MDR1 transport activity was examined using [^{3}H]digoxin, a typical substrate for MDR1, in the porcine kidney epithelial cell line LLC-PK$_1$ and in LLC-GA5-COL150, which was established by transfection of human *MDR1* cDNA into LLC-PK$_1$ cells (Tanigawara et al 1992; Ueda et al 1992). Comparison was made with amphotericin B and four azoles (ketoconazole, fluconazole, itraconazole and miconazole). The inhibitory effects of these six antifungal drugs on CYP3A4 metabolic activity were also evaluated based on nifedipine oxidation in human liver microsomes (Niwa et al 2003). Their rank order will be useful when selecting one of these antifungal drugs for patients taking drugs that are substrates for both CYP3A4 and MDR1.

Materials and Methods

Chemicals

Micafungin was supplied by Fujisawa Pharmaceutical Co. Ltd (Osaka, Japan). Other antifungals and colchicine were purchased from Wako Pure Chemical Industries Ltd (Osaka, Japan). Nifedipine and oxidized nifedipine were obtained from Sigma-Aldrich Co. (St Louis, MO, USA) and Ultrafine Chemicals (Manchester, UK), respectively. [^{3}H]Digoxin (865.8 GBq mmol^{-1}) and [methoxy-^{14}C]inulin (303 MBq mmol^{-1}) were purchased from New England Nuclear (Boston, MA, USA) and Amersham International plc (Buckinghamshire, UK), respectively. All other chemicals were obtained commercially or were of the highest grade requiring no further purification.

Inhibitory effects of antifungal drugs on CYP3A4 metabolic activity

Pooled human liver microsomes were obtained from XenoTech (Lenexa, KS, USA). Nifedipine oxidase was used as a probe for assessing the activity of CYP3A4. Nifedipine oxidase activity in the presence or absence of antifungals was determined as described previously (Niwa et al 2003). Briefly, the incubation mixture consisted of 0.05 mg mL^{-1} human liver microsomes, 10 μM nifedipine (around the expected Km), 2 mM NADP$^+$, 10 mM G6P, 5 mM magnesium chloride, 1 U mL^{-1} G6PDH, 100 mM phosphate buffer (pH 7.4), and antifungal drugs in a final volume of 500 μL. Incubation was carried out at 37°C for 10 min. The inhibitory effects were assessed as the 50% inhibitory concentration (IC50).

Culture of LLC-PK$_1$ and LLC-GA5-COL150 cells

LLC-GA5-COL150 cells were established by transfection of *MDR1* cDNA into porcine kidney epithelial LLC-PK$_1$ cells (Tanigawara et al 1992; Ueda et al 1992). LLC-PK$_1$ cells (220–230th passage) and LLC-GA5-COL150 cells (9–12th passage) were maintained in a culture medium consisting of Medium 199 (Dainippon Pharmaceutical Co., Ltd, Osaka, Japan) supplemented with 10% fetal bovine serum (lot no. AKH12368 or AMJ17247; HyClone, Logan, UT, USA) without antibiotics. For LLC-GA5-COL150 cells, 150 ng mL^{-1} of colchicine was added for the stable expression of MDR1. LLC-PK$_1$ (1.0×10^6 cells; 1.82×10^4 cells cm^{-2}) and LLC-GA5-COL150 (1.5×10^6 cells; 2.73×10^4 cells cm^{-2}) cells were seeded on plastic culture dishes (100 mm diameter) in 10 mL of the culture medium; monolayer cultures were grown in a humidified atmosphere of 5% CO_2/95% air at 37°C, and subcultured every 4 and 7 days, respectively, with 0.02% EDTA–0.05% trypsin solution (Invitrogen Corp., Carlsbad, CA, USA).

Inhibitory effects of antifungal drugs on MDR1 transport activity

The transcellular transport of [^{3}H]digoxin across LLC-PK$_1$ and LLC-GA5-COL150 cell monolayers was examined as described previously (Tanigawara et al 1992; Sakaeda et al 2002; Takara et al 2002a, b). Basal-to-apical transport and apical-to-basal transport were assayed independently. Cells were seeded on Transwell (cat. no. 3414; Corning Costar Corp., Cambridge, MA, USA) at a density of 2.0×10^6 cells/well (4.26×10^5 cells cm^{-2}) and 2.4×10^6 cells/well (5.11×10^5 cells cm^{-2}) for LLC-PK$_1$ and LLC-GA5-COL150 cells, respectively, and cultured in a humidified atmosphere of 5% CO_2/95% air at 37°C for 3 days. At 3 h before the start of transport experiments, the culture medium was renewed. Colchicine was not included even for LLC-GA5-COL150 cells. The transport experiment was initiated by replacement of the culture medium on the donor side with 2 mL of fresh culture medium containing [^{3}H]digoxin (100 nM, 18.5 kBq mL^{-1}) together with [methoxy-^{14}C]inulin (6.0 μM, 1.85 kBq mL^{-1}) and that on the receiver side with 2 mL of fresh culture medium. The monolayers were incubated at 37°C and samples (25 μL) of the culture medium on the receiver side were taken at 1, 2 and 3 h. Paracellular leakage estimated as the amount of [methoxy-^{14}C]inulin appearing on the receiver side was less than 0.4% h^{-1} of the initial amount of [methoxy-^{14}C]inulin on the donor side.

The effects of antifungal drugs on the transcellular transport of [^{3}H]digoxin were evaluated in LLC-GA5-COL150 cells by their addition to both sides of the monolayers at 1 h before the start of transport experiments, and by their addition to both sides of the monolayers when the transport experiments were started. The cells were exposed until the end of the transport experiments (i.e. for a total of 4 h). In the assessment, the net basal-to-apical transport rate was

calculated by subtracting the apical-to-basal transport from the basal-to-apical transport of [^{3}H]digoxin, and the rate of net basal-to-apical transport with an antifungal drug to that without any drug was calculated.

The level of radioactivity of the collected media was measured in 3 mL of ACS II (Amersham Biosciences Corp., Piscataway, NJ, USA) by liquid scintillation counting (LSC-5100; Aloka Co., Ltd, Tokyo, Japan). Data are presented as the percentage of the initial amount of total radioactivity added to the donor side.

Statistical analysis

All data are presented as the mean ± s.d. Statistical analysis was performed by one-way analysis of variance followed by Scheff's test (two-tailed), with $P < 0.05$ considered significant.

Results and Discussion

Figure 1 shows the effect of antifungal drugs on nifedipine oxidase activity. The IC50 values are given in Table 1. An inhibitory effect on CYP3A4 metabolic activity was found for ketoconazole, itraconazole and miconazole, with IC50 values of 11.7, 32.6 and 74.2 nM, respectively. Fluconazole and micafungin had only slight inhibitory effects and amphotericin B had no effect. The results are consistent with the those obtained using ciclosporin (Back & Tjia 1991), midazolam (von Moltke et al 1996), terfenadine (Shader et al 1996), zonisamide (Nakasa et al 1998) and diazepam (Tran et al 2002).

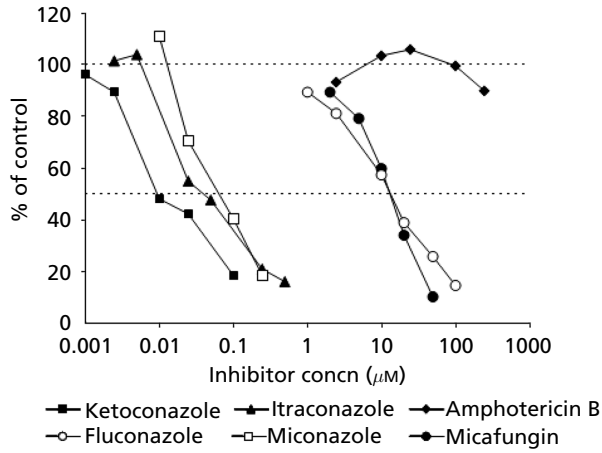

Figure 1 Effect of antifungal drugs on nifedipine oxidase activity in human liver microsomes.

Table 1 50% Inhibitory concentrations (IC50) of antifungal drugs for nifedipine oxidase activity in human liver microsomes

Antifungal agent	IC50 (μM)
Ketoconazole	0.0117
Itraconazole	0.0326
Fluconazole	13.1
Miconazole	0.0742
Micafungin	13.5
Amphotericin B	NC

NC, not calculated.

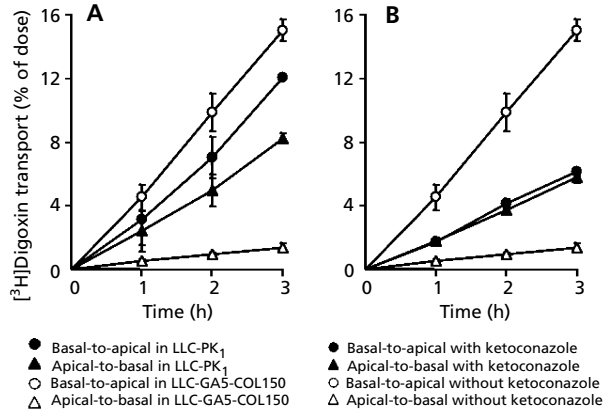

Figure 2 Transcellular transport of [^{3}H]digoxin across LLC-PK$_1$ and LLC-GA5-COL150 cell monolayers (A), and the effect of ketoconazole in LLC-GA5-COL150 cell monolayers (B). Each point represents the mean ± s.d. of at least three independent experiments.

Figure 2 shows the transepithelial transport of [^{3}H]digoxin across LLC-PK$_1$ and LLC-GA5-COL150 cell monolayers. Transport of [^{3}H]digoxin was time-dependent for at least 3 h, and the basal-to-apical transport in LLC-GA5-COL150 cell monolayers was greater than the apical-to-basal transport (Figure 2A). The basal-to-apical transport was increased and the apical-to-basal transport was decreased in LLC-GA5-COL150 cell monolayers as compared with LLC-PK$_1$ cell monolayers, showing that MDR1 acts as an efflux transporter at the apical membrane (Figure 2A). The basal-to-apical transport of [^{3}H]digoxin across LLC-GA5-COL150 cell monolayers was decreased, whereas the apical-to-basal transport was increased by ketoconazole at 50 μM (Figure 2B), suggesting the inhibition of MDR1-mediated transport of [^{3}H]digoxin by ketoconazole.

Figure 3 shows the effects of antifungal drugs on the net basal-to-apical transport of [^{3}H]digoxin across LLC-GA5-COL150 cell monolayers. MDR1-mediated transport of [^{3}H]digoxin was inhibited by ketoconazole and itraconazole at 5–50 μM. Miconazole showed slight inhibition at 25 and 50 μM. No effect was found for fluconazole and micafungin at 50 μM or amphotericin B at 25 μM. The effect of 50 μM amphotericin B could not be examined because of cytotoxicity. The inhibitory effect involves two processes: a direct inhibition of MDR1 and an intracellular accumulation in the cells. The apparent IC50 of ketoconazole for the net basal-to-apical transport of [^{3}H]digoxin was calculated to be 7.4 μM, being comparable with the value of 2.7 μM determined using the data of the basal-to-apical transport of rhodamine-123 in Caco-2 cells (von Moltke et al 2004).

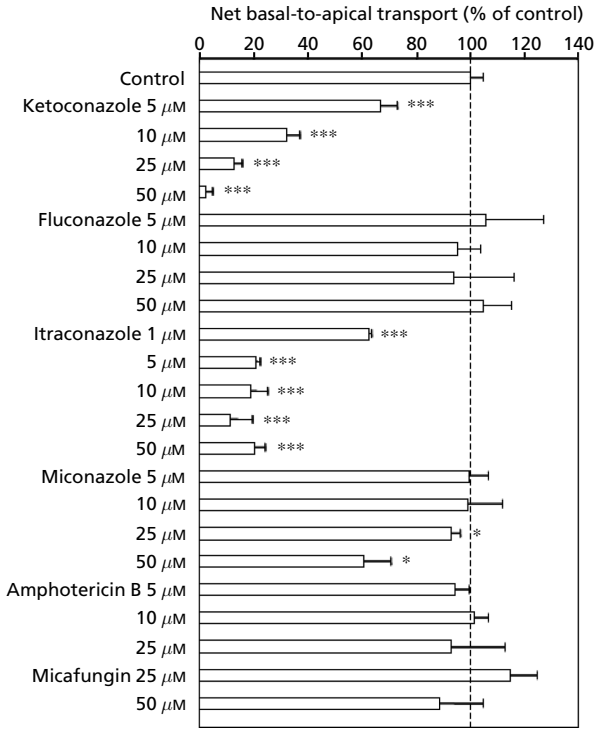

Figure 3 Effect of antifungal drugs on net basal-to-apical transport of [^{3}H]digoxin across LLC-GA5-COL150 cell monolayers. Each bar represents the mean ± s.d. $*P < 0.05$, $***P < 0.001$, significantly different compared with control.

In this study, we found that ketoconazole, itraconazole and, possibly, miconazole had inhibitory effects on both CYP3A4 metabolic activity and MDR1 transport activity; fluconazole and micafungin had only slight effects on CYP3A4, and amphotericin B had no effect. The pharmacokinetic profiles of two widely used immunosuppressants, ciclosporin and tacrolimus, were reported to be altered by co-administration of ketoconazole in transplant patients (Foradori et al 1998; McLachlan & Tett 1998; Moreno et al 1999). These interactions were discussed in terms of the inhibition of CYP3A4 metabolic activity, but the blocking of MDR1 transport activity would also be a factor, since ciclosporin and tacrolimus are also substrates for MDR1. Fluconazole has been reported to elevate the plasma concentration of midazolam in patients and healthy volunteers, and ciclosporin in patients (Olkkola et al 1996; Ahonen et al 1997, 1999; Sud et al 1999). On the other hand, no pharmacokinetic alteration of ciclosporin and tacrolimus was observed after co-administration of micafungin (Townsend et al 2002a, b). Protein binding in human serum is approximately 10% for fluconazole and 99.8% for micafungin. Although they have similar inhibitory effects on CYP3A4, fluconazole is thought to be distributed more to the liver, meaning that it is more likely to cause drug interaction. In the clinical setting, because the frequency of fungal infections is high in certain groups of patients, such as allogeneic haematopoietic stem-cell transplant recipients, lung transplant recipients and patients with AIDS (Moore & Chaisson 1996; Ribaud et al 1999; Denning 2003), antifungal drugs are likely to be used together with immunosuppressants or anti-HIV agents. Since protease inhibitors, including ritonavir, indinavir, nelfinavir and saquinavir, are also substrates for CYP3A4 and MDR1 (Fitzsimmons & Collins 1997; Kim et al 1998; Koudriakova et al 1998; Lee et al 1998), it is important to take into account the inhibitory effect on CYP3A4 when selecting antifungals. In addition, no information is available about the inhibitory effects of antifungal agents, including terbinafine, voriconazole, ravuconazole, posaconazole, anidulafungin and caspofungin, on MDR1 transport activity, although there have been several reports on how they influence CYP. Initially, terfenabine was reported not to have an inhibitory effect on CYP activity (Back & Tjia 1991) and not to change the disposition of triazolam in healthy volunteers (Varhe et al 1996). However, it has since been reported that terbinafine has a potent inhibitory effect on CYP2D6, with a Ki value of 0.2–0.25 μM (Vickers et al 1999), suggesting that it is likely to cause drug–drug interactions with substrates for CYP2D6. Voriconazole is a newly developed azole antifungal agent. Although information about its interaction has been limited so far, voriconazole reportedly raised the tacrolimus concentration in liver transplant recipients and inhibited the metabolism of tacrolimus, with an IC50 of 10.4 μg mL^{-1} (Venkataramanan et al 2002). Posaconazole was also reported to inhibit CYP3A4 in healthy subjects (Wexler et al 2004). Furthermore, another echinocandin antifungal, caspofungin, has been reported to have an inhibitory effect on CYP3A4 (Colburn et al 2004) but its impact in the clinical setting remains to be elucidated.

Figure 4 shows the relationship between the inhibitory effect on the activity of MDR1 (% of control of net basal-to-apical transport in the presence of 50 μM antifungal drugs) and metabolic activity of CYP3A4 (IC50 values for nifedipine oxidase). The inhibitory effects on CYP3A4 metabolic activity and MDR1 transport activity exhibited a positive correlation, as already pointed out by others (Wacher et al 1995, 1998, 2001; Wandel et al 1999; Benet et al 2003). However, the representative CYP3A4 substrates nifedipine and midazolam are not substrates for MDR1 (Venkatakrishnan et al 2000) and the inhibitory effects on CYP3A4 and MDR1 were variable (Perloff et al 2003). Further studies, such as X-ray analysis of protein crystals or physicochemical analysis of drug binding to the proteins, may explain the overlap of substrate or inhibitor specificity for CYP3A4 and MDR1 and any exceptions.

It is controversial whether substrates and/or inhibitors of MDR1 should be developed as new chemical entities (Lin & Yamazaki 2003). Drug interaction is a critical problem in the clinical setting as it often results in a higher frequency of adverse events. Therefore in the discovery stage of development, pharmaceutical companies tend to select compounds that are unlikely to cause drug interactions, and high-throughput screening systems must be established to determine inhibitory effects on CYP3A4 metabolic and MDR1 transport activities.

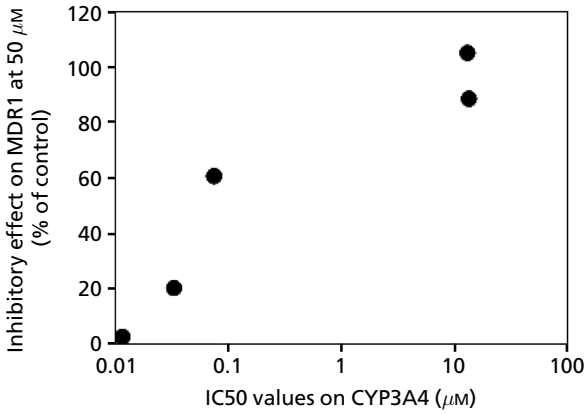

Figure 4 Relationship of inhibitory effect of antifungal drugs on CYP3A4 metabolic and MDR1 transport activities. The ratio of net basal-to-apical transport of [^{3}H]digoxin in the presence of 50 μM of antifungal drugs to that without any antifungal drugs was plotted against the logarithm of IC50 values for nifedipine oxidase activity. Amphotericin B was excluded because of its lack of an inhibitory effect.

Conclusion

We have demonstrated that in addition to amphotericin B, micafungin had little or no inhibitory effect on CYP3A4 metabolic and MDR1 transport activities, whereas ketoconazole, itraconazole and, possibly, miconazole showed both CYP3A4- and MDR1-mediated drug interactions. A positive correlation between the inhibitory effects on CYP3A4 and MDR1 activities was observed. The physicochemical mechanisms involved and impact on clinical treatment need to be studied further.

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