Substrate Specificity of Human Cytochrome P450 (CYP) 2C Subfamily and Effect of Azole Antifungal Agents on CYP2C8

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ABSTRACT - PURPOSE: The metabolic activities of aminopyrine *N*-demethylation and tolbutamide methylhydroxylation by the human hepatic cytochrome P450 (P450 or CYP) 2C subfamily were compared and the effects of azole antifungal agent on the drug-metabolizing activity of CYP2C8 were investigated. METHODS: Aminopyrine *N*-demethylation and tolbutamide methylhydroxylation by CYP2C8, CYP2C9, and CYP2C19 were determined by the previous reported methods. The effects of five azole antifungal agents, fluconazole, itraconazole, ketoconazole, miconazole, and voriconazole, on the aminopyrine *N*-demethylation activity by CYP2C8 were investigated. RESULTS: With regard to aminopyrine *N*-demethylation, CYP2C19 had the lowest Michaelis constant (K_m) and CYP2C8 had the highest maximal velocity (V_{max}) among the CYP2C subfamily members. The V_{max}/K_m values for CYP2C8 were the highest, followed by CYP2C19. For tolbutamide methylhydroxylation, the K_m and V_{max} for CYP2C19 were three and six times higher than the corresponding values for CYP2C9, and the V_{max}/K_m value for CYP2C19 was twice that for CYP2C9, whereas hydroxylated tolbutamide formed by CYP2C8 was not detected. Fluconazole, itraconazole, and voriconazole at a concentration of 2 or 10 μM neither inhibited nor stimulated CYP2C8-mediated aminopyrine *N*-demethylation activity at substrate concentrations around the K_m (5 mM). However, ketoconazole and miconazole noncompetitively inhibited CYP2C8-mediated aminopyrine *N*-demethylation with the inhibitory constant values of 1.98 and 0.86 μM, respectively. CONCLUSION: These results suggest that ketoconazole and miconazole might inhibit CYP2C8 clinically.

INTRODUCTION

Cytochrome P450s (P450 or CYP) are a superfamily of enzymes that catalyze the oxidation of a wide variety of endogenous and exogenous compounds, including drugs, carcinogens, and steroids (1,2). The CYP2C subfamily accounts for about 20% of all P450s present in human livers, and is one of the most important P450 subfamilies because it metabolizes more than 20% of all therapeutic drugs (3-5). The CYP2C subfamily in human liver comprises three members: CYP2C8, CYP2C9, and CYP2C19. Although the three CYP2C enzymes share more than 82% amino acid sequence identity, they have rather distinct substrate specificities (6-9).

Multiple drug therapy is a common therapeutic practice, particularly in patients with several diseases or conditions, and many drug–drug interactions involving metabolic inhibition have been reported. Because antibiotics and/or antifungals are co-administered to patients with severe illnesses, there is a possibility of drug-drug interactions (10-12). Macrolide antibacterials, especially troleandomycin and erythromycin, are inhibitors of CYP3A4-mediated metabolism (1,10), and quinolone antibacterial agents, including enoxacin and ciprofloxacin, inhibit the CYP1A2-mediated metabolism of drugs (1,11), although, oral cephalosporins such as cefixime and cefdinir neither inhibit nor stimulate the drug-metabolizing activities of P450s (12). Recently, we demonstrated that the penicillin-based antibiotics, amoxicillin and piperacillin, inhibited CYP2C8-mediated aminopyrine *N*-demethylation, although the interactions between these penicillin-based antibiotics and other drugs that are metabolized by P450s would not be clinically significant (13). We also found that CYP2C9 polymorphism affects the ability of azole antifungals to inhibit CYP2C9 (14). However, there have been minimal reports studying the effect of antifungal agents on the drug-metabolizing activity of CYP2C8 (15).

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Thus, in the present study, we compared the metabolic activities mediated by CYP2C8, CYP2C9, and CYP2C19 and investigated the effects of antifungals on CYP2C8-mediated aminopyrine *N*-demethylation.

METHODS

Materials

CYP2C8, CYP2C9, and CYP2C19 expressed in recombinant *Escherichia coli* (Bactosomes) were obtained from Cypex Ltd (Dundee, UK). Fluconazole, voriconazole, and methylhydroxylated tolbutamide were purchased from Tocris Bioscience (Bristol, UK), and tolbutamide from Sigma-Aldrich (MO, USA). Ketoconazole and miconazole were obtained from LKT Laboratories (St. Paul, MN, USA), and itraconazole from Tokyo Chemical Industry (Tokyo, Japan). All other reagents and organic solvents used were of the highest purity commercially available.

Determination of Human Drug-metabolizing P450 Activities

Aminopyrine *N*-demethylation and tolbutamide methylhydroxylation were determined in the presence or absence of antifungals, as described previously (16,17). The incubation mixture consisted of human P450, 1 mM of NADPH, 5 µl of methanol or 50–1000 µM of antifungal agents dissolved in methanol, and 100 mM of potassium phosphate buffer (pH 7.4) in a final volume of 0.5 ml. Because itraconazole at a concentration of above 200 µM was not dissolved in methanol, the itraconazole concentration in the incubation mixture was 2 µM. The P450 concentrations in the mixture were 10 (for aminopyrine *N*-demethylation) and 40 pmol/ml (for tolbutamide methylhydroxylation). Incubation times were 5 (for aminopyrine *N*-demethylation) or 30 min (for tolbutamide methylhydroxylation). In the preliminary experiments, the linearity of reaction with P450 concentrations and incubation times was confirmed for each CYP2C subfamily. All data were analyzed using the average of duplicate or triplicate determinations. Michaelis constants (K_m), maximal velocity (V_{max}), and inhibitory constants (K_i) were determined by performing Michaelis-Menten kinetics using nonlinear least squares regression by means of MULTI (18).

RESULTS

The kinetics of aminopyrine *N*-demethylation by CYP2C8, CYP2C9, and CYP2C19 were evaluated by fitting the values into the Michaelis–Menten plots. CYP2C19 had the lowest K_m and CYP2C8 had the highest V_{max} of the three CYP2C subfamily members. The V_{max}/K_m values for CYP2C8 were the highest, followed by CYP2C19 (Table 1).

Tolbutamide is a typical substrate of CYP2C9 *in vitro* and a probe drug used for *in vivo* drug interaction studies of CYP2C9, as recommended in the guidelines by the European Medicines Agency (EMA) (19) and U.S. Food and Drug Administration (FDA) (20). We recently demonstrated that the V_{max} and V_{max}/K_m values of CYP2C9.1 (wild type) and CYP2C9.2 (Arg144Cys) were similar and that CYP2C9.3 (Ile359Leu) had a higher K_m and a lower V_{max} than CYP2C9.1 and CYP2C9.2 (14). Thus, we compared the tolbutamide methylhydroxylation activities of CYP2C8 and CYP2C19 with that of CYP2C9.1. CYP2C19 had a three-times higher K_m and a six-times higher V_{max} than CYP2C9, and the V_{max}/K_m value for CYP2C19 was twice that of CYP2C9 (Table 1). On the other hand, methylhydroxylated tolbutamide catalyzed by CYP2C8 (40 pmol/ml) at a 100 µM substrate concentration was not detected (<0.1 µM) after 15 or 30 min of incubation, suggesting that the V_{max}/K_m was less than 1 µl/min/nmol.

The inhibitory effects of antifungals on aminopyrine *N*-demethylation mediated by CYP2C8 were investigated at a 5 mM substrate concentration, which is near the K_m obtained in this study and reported previously (16) (Fig. 1, A). At a 10 µM concentration, ketoconazole and miconazole inhibited the reaction by 61.2% and 76.2%, respectively, whereas fluconazole (10 µM), itraconazole (2 µM), and voriconazole (10 µM) had neither inhibitory nor stimulatory effects against CYP2C8. Ketoconazole and miconazole noncompetitively inhibited CYP2C8 activity and the estimated K_i values were 1.98 and 0.86 µM, respectively (Fig. 1, B and C).

DISCUSSION

Although paclitaxel 6α-hydroxylation and amodiaquine *N*-deethylation are typical metabolic reactions catalyzed by CYP2C8 (1,19,20), the metabolites are very expensive and thus rarely purchased. We previously demonstrated that several human hepatic P450s, including CYP2C8, CYP2C9, and CYP2C19, exhibit aminopyrine *N*-demethylation activity (16). Tolbutamide is a typical substrate of CYP2C9 *in vitro* and a probe drug used for *in vivo* drug interaction studies as recommended in the guidance by EMA (19) and by FDA (20); however, tolbutamide methylhydroxylation was

catalyzed by not only CYP2C9 but also CYP2C19 (8). In the present study, CYP2C19 showed a higher K_m and V_{max} than that shown by CYP2C9 and the V_{max}/K_m value for CYP2C19 was twice that of CYP2C9, whereas methylhydroxylated tolbutamide catalyzed by CYP2C8 was not detected. For aminopyrine *N*-demethylation, CYP2C19 had the lowest K_m and CYP2C8 and CYP2C19 had the highest V_{max} of the three CYP2C subfamilies; the V_{max}/K_m for CYP2C8 was the highest, followed by CYP2C19.

The present results are consistent with those in a previous report that aminopyrine *N*-demethylation activities at a 2 mM substrate concentration, which is near the K_m, were highest for CYP2C8 and CYP2C19 (15). These results suggest that aminopyrine *N*-demethylation activity could be used for *in vitro* drug-drug interaction studies on CYP2C8 (13) at a lower cost, although further comparative studies between aminopyrine *N*-demethylation and the typical metabolic reactions such as paclitaxel 6α-hydroxylation and amodiaquine *N*-deethylation might be necessary.

Table 1. Aminopyrine *N*-demethylation and tolbutamide methylhydroxylation activities mediated by CYP2C8, CYP2C9, and CYP2C19.

P450	Enzyme Expression	Aminopyrine *N*-demethylation			Tolbutamide methylhydroxylation		
		K_m (mM)	V_{max} (nmol/min/nmol)	V_{max}/K_m (µl/min/nmol)	K_m (µM)	V_{max} (nmol/min/nmol)	V_{max}/K_m (µl/min/nmol)
CYP2C8	*E. coli*	3.0 ± 0.4	185.6 ± 8.2	62.8	-	-	(<1)
CYP2C9.1	*E. coli*	10.9 ± 2.9	75.0 ± 6.7	6.9	98.1 ± 15.4 (14)	2.1 ± 0.1 (14)	21 (14)
CYP2C9.2	*E. coli*	-	-	-	130.4 ± 48.3 (14)	2.3 ± 0.3 (14)	18 (14)
CYP2C9.3	*E. coli*	-	-	-	334.8 ± 77.2 (14)	0.91 ± 0.09 (14)	2.7 (14)
CYP2C19	*E. coli*	1.3 ± 0.1	32.6±0.6	26.0	346 ± 23	13.7 ± 0.4	39
CYP2C8	Yeast	5.3 (16)	188 (16)	36 (16)	-	-	-
CYP2C19	Yeast	0.31 (16)	105 (16)	333 (16)	-	-	-

Values are mean ± S.D. of the data set using a nonlinear kinetic analysis from mean values obtained in duplicate at each substrate concentration. The 16 in parentheses is the reference for the yeast data.

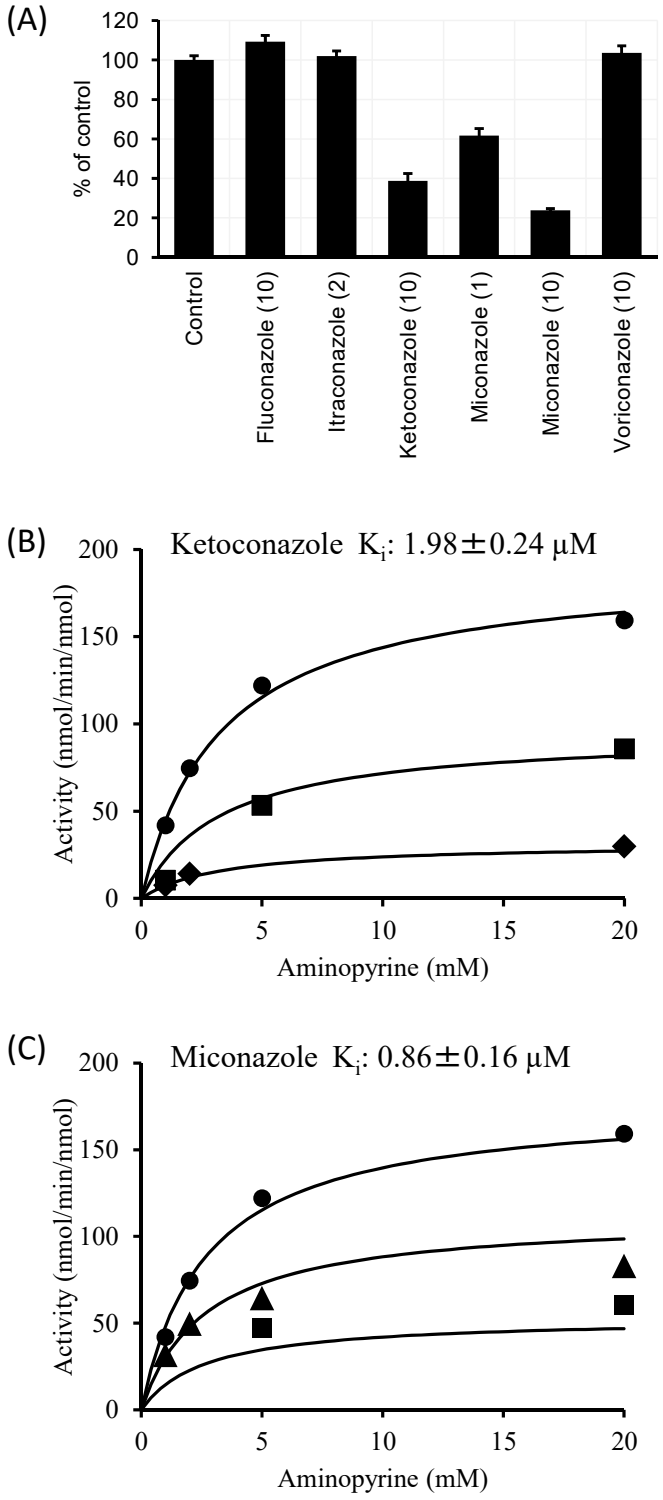

Figure 1. Inhibitory effects of azole antifungals against CYP2C8-mediated aminopyrine *N*-demethylation activity. Inhibitory effects of azole antifungals (A), ketoconazole (B) and miconazole (C). (A) Data are means ± S.D. (n=3). Values in parentheses are the concentrations of antifungals in the incubation mixture (μM). (B), (C): All data were analyzed using the average of duplicate determinations. K_i values and the standard deviations as indexes of the precision of the calculated parameters were determined by performing Michaelis-Menten kinetics using nonlinear least squares regression by means of MULTI (18). ●：0 μM, ▲：0.5 μM, ■：2 μM, ◆：10 μM.

In the present study, the relative affinity and/or strength, demonstrated as kinetic parameters such as K_m, V_{max}, and K_m/V_{max}, of aminopyrine *N*-demethylation and tolbutamide methylhydroxylation among CYP2C8, CYP2C9, and CYP2C19 were reaction-dependent. We have previously reviewed the comparison of these kinetic parameters of the CYP2C subfamily members for 74 metabolic reactions of 45 substrates (9). In these reactions, the kinetic behaviors of these CYP2C subfamilies depend on the metabolic reaction, although the ratios of V_{max}/K_m for CYP2C19/CYP2C9 and CYP2C8/CYP2C19, but not for CYP2C8/CYP2C9, were more closely correlated with K_m values than with V_{max} values. This suggests that the differences in affinity may be more important than the differences in capacity for the substrate/reaction specificity of CYP2C subfamilies, especially for CYP2C19 (9). Further investigation, for instance, using three-dimensional structural analysis such as molecular docking simulation (21) is required.

Ketoconazole and miconazole noncompetitively inhibited aminopyrine *N*-demethylation catalyzed by CYP2C8, whereas no inhibition was observed for fluconazole, itraconazole, and voriconazole (Fig. 1). Ketoconazole has been recognized as a weak inhibitor of CYP2C8 as listed in US FDA Guidance for Industry - Drug Interaction Studies (2012) (22), however, there were few reports in terms of the inhibition of CYP2C8 by miconazole (15). It has been established that imidazole and triazole compounds, including azole antifungals, reversibly inhibit drug-metabolizing P450s by heme-nitrogen interactions (23,24). Fluconazole can exhibit competitive, noncompetitive, or mixed-type inhibition against CYP3A4 (14,15,25-28). Itraconazole competitively inhibited CYP3A4 (15,17,27,29). On the other hand, it has been reported that fluconazole and voriconazole exhibited mixed-type inhibition and competitive inhibition, respectively, against CYP2C9 (14,15,24,30). We recently reported that voriconazole noncompetitively inhibited tolbutamide hydroxylation catalyzed by CYP2C9.1 (wild type) and CYP2C9.3, whereas fluconazole and voriconazole competitively inhibited the hydroxylation catalyzed by CYP2C9.2 and fluconazole competitively inhibited the reaction catalyzed by CYP2C9.1 (14). Thus, the inhibition type seems to be different among substrates, inhibitors (azole antifungals), P450s, and/or other experimental conditions.

The K_i values of ketoconazole for CYP2C8 obtained in the present study (1.98 μM) was lower than the reported IC_{50} value (28 μM), at a substrate concentration of nearly K_m toward CYP2C19-mediated *S*-mephenytoin 4′-hydroxylation activity in human liver microsomes (HLM) (31). For miconazole, the K_i values for CYP2C8 obtained in the present study (0.86 μM) was comparable to the reported IC_{50} value (0.33 μM), at a substrate concentration of nearly K_m toward CYP2C19-mediated *S*-mephenytoin 4′-hydroxylation activity in HLM (32). When the substrate concentration is lower than the K_m value, the ratio of intrinsic clearance (CL_{int}) in the presence and absence of the inhibitor can be expressed by the following equation, independent of the inhibition type, except in the case of uncompetitive inhibition (33-35):

$$CL_{int}\ (+\ \text{Inhibitor}) / CL_{int}\ (-\ \text{Inhibitor}) = 1 / (1 + I_u/K_i)$$

where I_u is the unbound concentration of the inhibitor. Both ketoconazole and miconazole were reported to increase the plasma concentrations of CYP2C19 substrates such as omeprazole and *S*-mephenytoin, respectively, to more than 1.4 fold (36,37). Thus, these two antifungals might affect drug pharmacokinetics and CYP2C8-mediated drug metabolism. In this regard, however, drugs predominantly metabolized by CYP2C8 alone are not known. More careful administration of azole antifungals and CYP2C8 substrates is required, although the EMA has already recommended the suspension of marketing authorizations for oral ketoconazole (38), and the FDA limits the usage of Nizoral (ketoconazole) oral tablets due to potentially fatal liver injury, and the risk of drug interactions and adrenal gland problems (39). Topical formulations of ketoconazole (such as creams, ointments, and shampoos) seem to be able to continue to be used because the amount of ketoconazole absorbed through the body is very low in these formulations (38).

In conclusion, the present study suggests that ketoconazole and miconazole but not fluconazole, itraconazole, and voriconazole, might inhibit CYP2C8 clinically, although further clinical drug-drug interaction studies are required.

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