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Characterization of human cytochrome P450 enzymes involved in the metabolism of cilostazol

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Running title: Cilostazol metabolism by human CYPs

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Abbreviations:CYP, cytochrome P450; HPLC, high-performance liquid chromatography; CLint, intrinsic clearance; SNP, single nucleotide polymorphism;

ABSTRACT:

Cilostazol (OPC-13013 or 6-[4-(1-cyclohexl-1H-tetrazol-5-yl)butoxy]-3,4-dihydro-2(1H)-quinolinone) is widely used as an antiplatelet vasodilator agent. *In vitro*, the hydroxylation of the quinone moiety of cilostazol to OPC-13326 is the predominant route, and the hydroxylation of the hexane moiety to OPC-13217 is the second most predominant route. This study was carried out to identify and kinetically characterize the human cytochrome P450 (CYP) isozymes responsible for the formation of the 2 major metabolites of cilostazol, namely, OPC-13326 and OPC-13217. In *in vitro* studies using 14 recombinant human CYP isozymes, CYP1A1, CYP1A2, CYP1B1, CYP2A6, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, CYP2E1, CYP2J2, CYP3A4, CYP3A5, and CYP4A11, cilostazol was metabolized to OPC-13326 mainly by CYP3A4 (Km = 5.26 µM, intrinsic clearance (CLint) = 0.34 µL/pmol CYP/min), CYP1B1 (Km = 11.2 µM, CLint = 0.03 µL/pmol CYP/min), and CYP3A5 (Km = 2.89 µM, CLint = 0.05 µL/pmol CYP/min) and to OPC-13217 mainly by CYP3A5 (Km = 1.60 µM, CLint = 0.57 µL/pmol CYP/min), CYP2C19 (Km = 5.95 µM, CLint = 0.16 µL/pmol CYP/min), CYP3A4 (Km = 5.35 µM, CLint = 0.10 µL/pmol CYP/min), and CYP2C8 (Km = 33.8 µM, CLint = 0.009 µL/pmol CYP/min). The present study showed that the 2 major metabolites of cilostazol *in vitro*, namely, OPC-13326 and OPC-13217, are mainly catalyzed by CYP3A4 and CYP3A5, respectively.

Cilostazol is a cyclic nucleotide phosphodiesterase type-3 inhibitor with antiplatelet, antithrombotic, and vasodilating properties. It also exhibits antiproliferative effects on smooth muscle cells (Tsuchikane et al., 1997) and has beneficial effects on high-density lipoprotein cholesterol and triglyceride levels *in vivo* (Elam et al., 1998).

The major pharmacologically active metabolites of cilostazol identified in the plasma of humans are OPC-13015 and OPC-13213 (Bramer et al., 1999). OPC-13015 is three times more potent than cilostazol with regard to inhibition of platelet aggregation, whereas OPC-13213 is three times less potent than cilostazol (Okuda et al., 1993). On the other hand, *in vitro*, the hydroxylation of the quinone moiety of cilostazol to OPC-13326 is the predominant route, and the hydroxylation of the hexane moiety to OPC-13217 is the second most predominant route (Fig. 1) (Akiyama et al., 1985). The pharmacological potency of both OPC-13326 and OPC-13217 remains unclear. An early *in vitro* study with human liver microsomes and selective CYP inhibitors indicated that cilostazol is extensively metabolized to OPC-13326 via the hepatic enzyme CYP3A and to OPC-13217 via CYP2C19 (Abbas et al., 2000). It has also been reported that the other CYPs—CYP1A2 and CYP2D6—may be partially involved in the metabolism of cilostazol (Abbas et al., 2000). Furthermore, significant drug interactions are observed when cilostazol is coadministered with other agents that inhibit CYP3A (e.g., erythromycin) (Suri et al., 1999a) or CYP2C19 (e.g., omeprazol) (Suri and Bramer, 1999b).

To our knowledge, the specific CYP isozymes (e.g., CYP3A4 and/or CYP3A5) involved in the metabolism of cilostazol have not yet been identified by using individual CYP isozymes, and the enzyme kinetic parameters such as Km, Vmax, and intrinsic clearance (CLint) have not been determined. The objectives of this study were to identify and kinetically characterize the human CYP isozymes responsible for the 2 major metabolites of cilostazol, namely, OPC-13326 and OPC-13217, by using recombinant human CYP isozymes.

Materials and Methods

Reagents and Chemicals.

Cilostazol, OPC-13015, OPC-13213, OPC-13217, OPC-13325, and OPC-3930 were provided by Otsuka Pharmaceutical Company, Tokushima, Japan. NADPH was purchased from Oriental Yeast Co. (Tokyo, Japan). Microsomes prepared from baculovirus-infected insect cells expressing CYP1A2, CYP2B6, CYP2C9, CYP2C19, CYP2D6, CYP2E1, CYP3A4, and CYP3A5 were obtained from BD Gentest (MA, USA). Microsomes prepared from baculovirus-infected insect cells expressing CYP1A1, CYP1B1, CYP2A6, CYP2C8, CYP2J2, and CYP4A11 were obtained from Invitrogen (CA, USA). All recombinant CYPs had been coexpressed with NADPH P450 oxidoreductase. Recombinant CYP2A6, CYP2C8, and CYP2J2 were also coexpressed with cytochrome b5. The other chemicals and reagents used in this study were of research-grade.

Enzyme Assay.

In vitro screening of cilostazol with the 14 human CYP baculosomes—CYP1A1, CYP1A2, CYP1B1, CYP2A6, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, CYP2E1, CYP2J2, CYP3A4, CYP3A5, and CYP4A11—was performed using a constant amount of cytochrome P450 (5 pmol/tube) and 0.2–50 µM cilostazol. All incubations were carried out for 20 min and the incubated mixture consisted of the following: baculosomes, 3.3 mM magnesium chloride, 1.3 mM NADPH, and cilostazol in 250 µL of 100 mM potassium phosphate buffer (pH 7.4). After preincubation at 37°C for 1 min, the reactions were initiated by the addition of substrate; it was then allowed to proceed at 37°C, and terminated with ice-cold acetonitrile. OPC-3930 was added as an internal standard (Tata et al., 2001). The incubation mixtures were centrifuged at 14,000 rpm for 5 min. After centrifugation, the supernatant was transferred into vials. The samples were stored at 4°C until analysis and 50µL of the sample was injected into the HPLC system described below for determination of cilostazol and its metabolite concentrations.

Analytical Procedures.

The system used for analysis consisted of an Alliance 2695 separation module (Waters, MA, USA), 2996 Photodiode Array Detector (254 nm; Waters), and a Sunfire C18 column (particle size of 5 µm, 4.6 mm × 150 mm; Waters). The column temperature was maintained at 40°C. The mobile phase, consisting of water with 25% acetonitrile (A) and 60% acetonitrile (B), was operated at a constant flow rate (1.0 mL/min). The elution gradient was a linear increase to 68% B in 20 min. The retention times of OPC-13213, OPC-13217, OPC-13326, internal standard (OPC-3930), OPC-13015, and cilostazol in this analytical condition were 5.6 min, 6.2 min, 12.3 min, 12.8 min, 15.3 min, and 17.4 min, respectively.

Calculations.

The apparent Vmax and Km parameters were calculated using a nonlinear regression program, SigmaPlot (HULINKS, Tokyo), fitted to the Michaelis-Menten and Eadie –Hofstee plots. The means of the data were obtained by at least 3 determinations.

Results and Discussion

The metabolic activities of the 14 human recombinant CYP isozymes—CYP1A1, CYP1A2, CYP1B1, CYP2A6, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, CYP2E1, CYP2J2, CYP3A4, CYP3A5, and CYP4A11—toward cilostazol were evaluated. As shown in Fig. 2, CYP3A4 was found to be the most efficient for the production of OPC-13326, while CYP3A5 and CYP2C19 were the most efficient for the production of

OPC-13217. CYP1A1, CYP1A2, CYP1B1, CYP2C19, CYP2D6, CYP2E1, and CYP3A5 showed detectable activity for the production of OPC-13326, while CYP2B6, CYP2C8, CYP2D6, CYP2J2, and CYP3A4 showed detectable activity for the production of OPC-13217.

Among those tested, the 5 most efficient human CYP isozymes—CYP1B1, CYP3A4, CYP3A5, CYP2C8, and CYP2C19—for cilostazol metabolism were selected to further kinetically characterize their metabolic activity. The kinetics of both OPC-13326 and OPC-13217 obtained from cilostazol by all the tested recombinant CYP isozymes were consistent with the Michaelis-Menten model. Furthermore the Eadie-Hofstee plots for the formation of metabolites showed a monophasic profile in all recombinant CYP isozymes tested (data not shown). As shown in Table 1, CYP3A4 was identified as the most efficient isozyme for generating OPC-13326 with the highest Vmax values for the metabolite. The Km, Vmax, and CLint for the production of OPC-13326 from cilostazol by the CYP3A4 isozyme were 5.26 µM, 1.93 pmol/pmol CYP/min, and 0.34 µL/pmol CYP/min, respectively. CYP3A5 had the highest affinity (i.e., the lowest Km values) (Km = 1.60 µM), and CYP2C19 had the highest Vmax (Vmax = 0.94 pmol/pmol CYP/min) for cilostazol in the production of OPC-13217. This accounts for their greater CLint values relative to the other CYP isozymes.

The metabolism of cilostazol was examined by using microsomes expressing individual human CYP enzymes in order to kinetically characterize the CYP enzymes involved in the metabolic pathways of cilostazol. The results presented above indicate a major contribution of CYP3A4 to the formation of OPC-13326 from cilostazol and of CYP3A5 and CYP2C19 to the formation of OPC-13217. Although it was thought that cilostazol would be metabolized to OPC-13217 mainly via CYP2C19 (Abbas et al., 2000), CYP3A5 should be the key enzyme involved in the formation of OPC-13217 from cilostazol.

Interestingly, both CYP3A5 (Kuehl et al., 2001; Xie et al., 2004; Roy et al., 2005) and CYP2C19 (de Morais et al., 1994a; de Morais et al., 1994b) are genetically polymorphic enzymes. For CYP3A5 variant alleles, the CYP3A5*3 allele is characterized by an A to G single nucleotide polymorphism (SNP) in intron 3 of the CYP3A5 gene that creates a cryptic consensus splice site and exon 3B and causes a splicing defect (Kuehl et al., 2001). The allelic frequency of the inactive CYP3A5*3 has been reported to be 74% in Japanese, 93% in Caucasians, and 32% in African Americans (Hiratsuka et al., 2002; Roy et al., 2005). Numerous functional polymorphisms in the CYP2C19 gene have also been identified; some alleles (e.g., CYP2C19*2 and CYP2C19*3) are associated with poor metabolism of CYP2C19 substrate drugs (Ieiri et al., 1996). The individuals with poor CYP2C19 metabolism comprise 20% of the Japanese and 3% of Caucasians (Kimura et al., 1998). If CYP3A5 and CYP2C19 have a significant role in the metabolism of cilostazol *in vivo* as well as *in vitro*, the individuals with poor CYP3A5 and/or CYP2C19 metabolism might have higher plasma cilostazol concentrations than those who can extensively metabolize via CYP3A5 and/or CYP2C19. However, since both OPC-13015 and OPC-13213 are active, it would be difficult to assess the clinical outcome in subjects that polymorphically express CYP3A5 or CYP2C19 without an *in vivo* data. In order to understand the mechanistic basis of our findings further, it would be of great value to clinically examine the relationship between the genotypes of both CYP3A5 and CYP2C19 and the plasma concentration of cilostazol and its metabolites.

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Footnotes

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Legends for figures

Fig. 1. Metabolic pathways of cilostazol involving human hepatic CYP isozymes

Fig. 2. Biotransformation of cilostazol via OPC-13323 (A) and OPC-13217 (B) by the cDNA-expressed human CYP isozymes. Cilostazol (50 µM) was metabolized by individual CYP isozymes (20 pmol/mL) with NADPH at 37°C for 20 min. The specific activities are expressed as picomoles of product generated per picomole of CYP isozyme per minute. The data shown are the mean ± SEM. (n = 3).

TABLE 1

Kinetic data determined during in vitro metabolism of cilostazol to OPC-13326 and OPC-13217 using human recombinant CYP isozymes

Metabolites	CYP isozymes	Km (μM)	Vmax (pmol/pmol CYP/min)	CLint (Vmax/Km) (μL/pmol CYP/min)
OPC-13326	CYP1B1	11.2	0.34	0.03
	CYP3A4	5.6	1.93	0.34
	CYP3A5	2.9	0.14	0.05
OPC-13217	CYP2C8	33.8	0.30	0.01
	CYP2C19	6.0	0.94	0.16
	CYP3A4	5.4	0.53	0.10
	CYP3A5	1.6	0.92	0.57

Figure 1

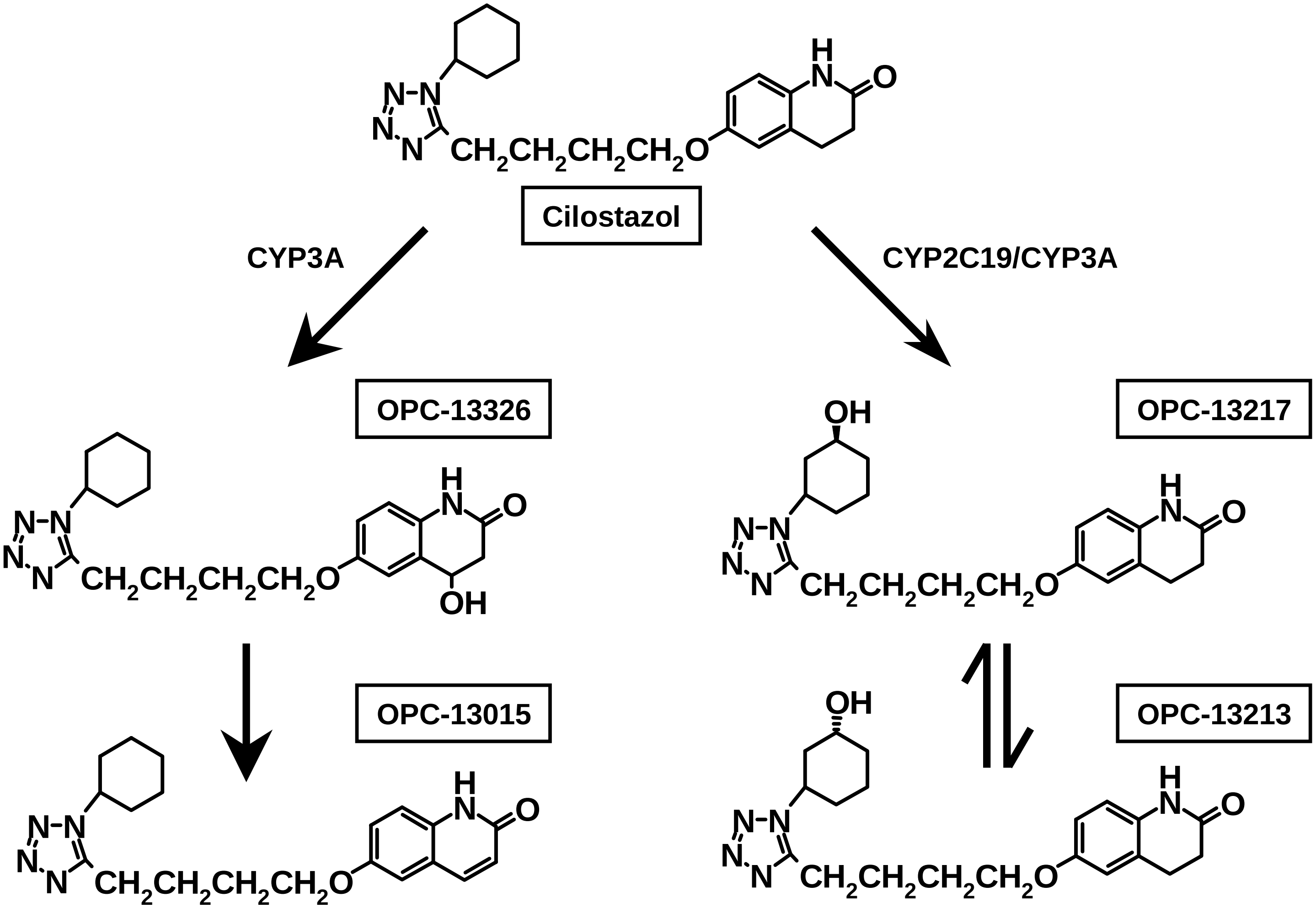
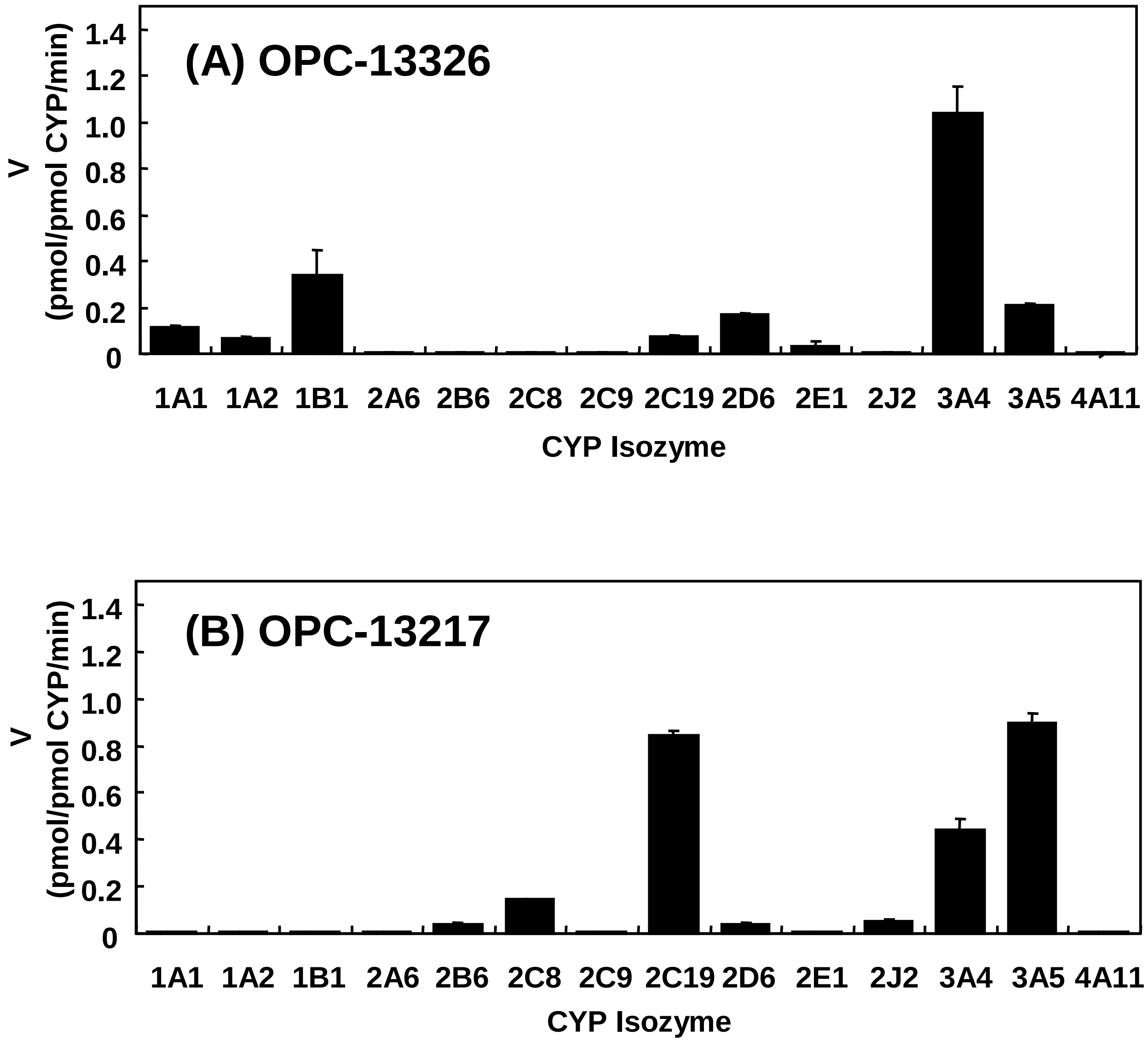

Cilostazol
CYP3A
CYP2C19/CYP3A
OPC-13326
OPC-13217
OPC-13015
OPC-13213
N-N
N
N
H
N
O
OH
CH2CH2CH2CH2O

Figure 2


(A) OPC-13326
V
(pmol/pmol CYP/min)
0
0.2
0.4
0.6
0.8
1.0
1.2
1.4
1A1
1A2
1B1
2A6
2B6
2C8
2C9
2C19
2D6
2E1
2J2
3A4
3A5
4A11
CYP Isozyme
(B) OPC-13217
V
(pmol/pmol CYP/min)
0
0.2
0.4
0.6
0.8
1.0
1.2
1.4
1A1
1A2
1B1
2A6
2B6
2C8
2C9
2C19
2D6
2E1
2J2
3A4
3A5
4A11
CYP Isozyme