ABSTRACT:
Cilostazol (OPC-13013; 6-[4-(1-cyclohexyl-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 [6-[4-(1-cyclohexyl-1H-tetrazol-5-yl)butoxy]-3,4-dihydro-4-hydroxy-2(1H)-quinolinone], 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 (P450) isozymes responsible for the formation of the two major metabolites of cilostazol, namely, OPC-13326 and OPC-13217 [3,4-dihydro-6-[4-[1-((cis-4-hydroxycyclohexyl)-1H-tetrazol-5-yl)butoxy]-2(1H)-quinolinone]]. In vitro studies using 14 recombinant human P450 isozymes, CYP1A1, CYP1A2, CYP1B1, CYP2A6, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, CYP2E1, CYP2J2, CYP3A4, CYP3A5, CYP4A11, cilostazol was metabolized to OPC-13326 mainly by CYP3A4 (Km = 5.26 μM, intrinsic clearance (Clint) = 0.34 μl/pmol P450/min), CYP1B1 (Km = 11.2 μM, Clint = 0.03 μl/pmol P450/min), and CYP3A5 (Km = 2.89 μM, Clint = 0.05 μl/pmol P450/min) and to OPC-13217 mainly by CYP3A4 (Km = 1.60 μM, Clint = 0.57 μl/pmol P450/min), CYP2C19 (Km = 5.95 μM, Clint = 0.16 μl/pmol P450/min), CYP3A4 (Km = 5.35 μM, Clint = 0.10 μl/pmol P450/min), and CYP2C8 (Km = 33.8 μM, Clint = 0.009 μl/pmol P450/min). The present study showed that the two 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, antiatherogenic, 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 3 times more potent than cilostazol with regard to inhibition of platelet aggregation, whereas OPC-13213 is 3 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 P450 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 P450s, 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., 1999) or CYP2C19 (e.g., omeprazole) (Suri and Bramer, 1999).

To our knowledge, the specific P450 isozymes (e.g., CYP3A4 and/or CYP3A5) involved in the metabolism of cilostazol have not yet been identified by using individual P450 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 P450 isozymes responsible for the two major metabolites of cilostazol, namely, OPC-13326 and OPC-13217, by using recombinant human P450 isoforms.

Materials and Methods
Reagents and Chemicals. Cilostazol, OPC-13015, OPC-13213, 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 (Woburn, MA). Microsomes prepared from baculovirus-infected insect cells expressing CYP1A1, CYP1B1, CYP2A6, CYP2C8, CYP2J2, and CYP4A11 were obtained from Invitrogen (Carlsbad, CA). All recombinant P450s had been coexpressed with cytochrome P450s (e.g., omeprazole) (Suri and Bramer, 1999).

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Abbreviations: P450, cytochrome P450; Clint, intrinsic clearance; SNP, single-nucleotide polymorphism; cilostazol, OPC-13013, 6-[4-(1-cyclohexyl-1H-tetrazol-5-yl)butoxy]-3,4-dihydro-2(1H)-quinolinone; OPC-13326, 6-[4-(1-cyclohexyl-1H-tetrazol-5-yl)butoxy]-3,4-dihydro-4-hydroxy-2(1H)-quinolinone; OPC-13217, 3,4-dihydro-6-[4-[1-(cis-4-hydroxycyclohexyl)-1H-tetrazol-5-yl]butoxy]-2(1H)-quinolinone; OPC-3930, 6-[3-(1-cyclohexyl-1H-tetrazol-5-yl)propoxy-2(1H)-quinolinone].
Enzyme Assay. In vitro screening of cilostazol with the 14 human P450 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 to 50 μM cilostazol. All incubations were carried out for 20 min, and the incubated mixture consisted of 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 was 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 50 μl of the sample was injected into the high-performance liquid chromatography 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, Milford, MA), 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 $V_{\text{max}}$ and $K_m$ parameters were calculated using a nonlinear regression program, SigmaPlot (HULINKS, Tokyo, Japan), 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 P450 isoforms 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, whereas CYP3A5 and CYP2C19 were the most efficient for the production of OPC-13217. CYP1A1, CYP1A2, CYP1B1, CYP2C9, CYP2C19, CYP2D6, CYP2E1, and CYP3A5 showed detectable activity for the production of OPC-13326, and CYP2B6, CYP2C8, CYP2D6, CYP2J2, and CYP3A4 showed detectable activity for the production of OPC-13217.

Among those tested, the five most efficient human P450 isoforms, 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 P450 isoforms were consistent with the Michaelis-Menten model. Furthermore the Eadie-Hofstee plots for the formation of metabolites showed a monophasic profile in all recombinant P450 isoforms tested (data not shown). As shown in Table 1, CYP3A4 was identified as the most efficient isoform for generating OPC-13326, with the highest $V_{\text{max}}$ values for the metabolite. The $K_m$, $V_{\text{max}}$, and $CL_{\text{int}}$ values for the production of OPC-13326 from cilostazol by the CYP3A4 isoform were 5.26 μM, 1.93 pmol/pmol P450/min, and 0.34 μl/pmol P450/min, respectively. CYP3A5 had the highest affinity (i.e., the lowest $K_m$ values) ($K_m = 1.60 \mu M$), and CYP2C19 had the highest $V_{\text{max}}$ ($V_{\text{max}} = 0.94 \text{ pmol/pmol P450/min}$) for cilostazol in the production of
The metabolism of cilostazol was examined by using microsomes expressing individual human P450 enzymes to kinetically characterize the P450 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 CYP3A5 and/or CYP2C19, however, because both OPC-13015 and OPC-13213 are active, it would be difficult to assess the clinical outcome in subjects who polymorphically express CYP3A5 or CYP2C19 without in vivo data. 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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### References


