Effect of Probucol on Cytochrome P450 Activities in Human Liver Microsomes

Ken UMEHARA,* Yoshihiko SHIMOKAWA, and Gohachiro MIYAMOTO

Tokushima Research Institute, Otsuka Pharmaceutical Co., Ltd.; 463–10 Kagasuno, Kawauchi-cho, Tokushima 771–0192, Japan. Received February 8, 2002; accepted May 17, 2002

The effects of probucol, a cholesterol-lowering agent, on several cytochrome P450 (CYP) isoform-specific reactions in human liver microsomes were investigated to predict drug interactions with probucol in vivo from in vitro data. The following eight CYP catalytic reactions were used in this study: CYP1A1/2-mediated 7-ethoxylresoerufin O-deethylation, CYP2A6-mediated coumarin 7-hydroxylation, CYP2B6-mediated 7-benzoylresorufin O-debenzylazion, CYP2C8/9-mediated tolbutamide methylhydroxylation, CYP2C19-mediated S-mephenytoin 4'-hydroxylation, CYP2D6-mediated bufuralol 1'-hydroxylation, CYP2E1-mediated chlorozoxazine 6-hydroxylation, and CYP3A4-mediated testosterone 6β-hydroxylation. Probucol had neither stimulatory nor inhibitory effects on CYP1A1/2, 2A6, 2B6, 2C8/9, 2C19, 2D6, 2E1, and 3A4 activities at concentrations up to 300 μM, indicating that probucol, at the expected therapeutic concentrations, would not be predicted to cause clinically significant interactions with other CYP-metabolized drugs.

Key words probucol; in vitro drug–drug interaction; human liver microsome

Probucol is a marketed cholesterol-lowering agent1,2 that has been reported to be active in patients with mild and severe forms of hypercholesterolemia.3,4 The antioxidant activity of probucol inhibits the formation of oxidatively modified low-density lipoproteins (LDL) and reduces atherosclerosis in animals.5—10 Probucol is metabolized in humans and animals, and three major metabolites have been structurally identified as spiroquinone, diphenoquinone, and bisphenol (Fig. 1).10,11 Interestingly, bisphenol is also an antioxidant, being oxidized to diphenoquinone when acting as such. Therefore the antioxidant activity is continuously regenerated in vivo.

Most oxidative metabolism of drugs is catalyzed by cytochrome P450 (CYP) enzymes, which comprise a large family of hemoproteins.12,13 More than 15 isoforms have been identified in human liver, and several forms play important roles in the metabolism of drugs.14 It is important to identify the enzymes (CYP isoforms) responsible for the metabolism of drugs and to investigate further whether a drug has an inhibitory effect on the catalytic activity of each CYP isoform to consider possible drug interactions. However, it is unclear which enzymes catalyze the metabolism of probucol and whether probucol has an inhibitory effect on the catalytic activity of CYP isoforms. In this study, we examined the characteristic properties of probucol on the catalytic activities of the CYP isoforms, CYP1A1/2, CYP2A6, CYP2B6, CYP2C8/9, CYP2C19, CYP2D6, CYP2E1, and CYP3A4 using specific reaction probes for the enzymes.

MATERIALS AND METHODS

Chemicals Probucol was supplied by Otsuka Pharmaceutical Co., Ltd. (Tokyo, Japan). Coumarin, tolbutamide, chlorozoxazine, glucose-6-phosphate disodium salt: hydrate, and β-NADP sodium salt were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Other chemicals were obtained from the following sources: 7-ethoxylresorufin, 4-hydroxytolbutamide, S-(+)-mephenytoin, (±)-4'-hydroxymephenytoin, 1'-hydroxybufuralol maleate salt, (±)-bufuralol hydrochloride salt, 6-hydroxychlorozoxazine, and 6β-hydroxytestosterone from Salford Ultrafine Chemical & Research Ltd. (Guildhall Close, Manchester, U.K.); 7-benzoylresorufin from Molecular Probes (Eugene, OR, U.S.A.); 7-hydroxycoomarin from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan); resorufin, testosterone, and magnesium chloride hexahydrate from Wako Pure Chemical Industries, Ltd. (Osaka, Japan); glucose-6-phosphate dehydrogenase (approximately 350 U/mg, 1 mg/ml) from Boehringer Mannheim GmbH (Mannheim, Germany). All other reagents and solvents were of high analytical grade.

Liver Microsome Preparation Pooled human liver microsomes from 10 donors were prepared at the Biomedical
Research Institute, Human and Animal Bridge Discussion Group (HAB, Chiba, Japan). Human liver samples were legally procured from the National Disease Research Interchange (NDRI, PA, U.S.A.) through the international partnership between the NDRI and HAB. The study was conducted in accordance with the Declaration of Helsinki. Human livers were homogenized with 0.25 M sucrose containing 3 mM Tris and 0.1 mM EDTA (pH 7.4), and microsomes were isolated by differential centrifugation using the common method. Washed microsomes were resuspended in 100 mM Tris–HCl buffer (pH 7.4) containing 1 mM EDTA and 20% glycerol at protein concentrations of 20 mg/ml.

**Determination of Human CYP Activities**

7-Ethoxyresorufin O-deethylase activity by CYP1A1/2, coumarin 7-hydroxylase activity by CYP2A6, 7-benzoxylresorufin O-debenzylation activity by CYP2B6, tolbutamide methylhydroxylation activity by CYP2C8/9, S-mephenytoin 4'-hydroxylation activity by CYP2C19, bufuralol 1'-hydroxylation activity by CYP2D6, chlorozoxazone 6-hydroxylation activity by CYP2E1, and testosterone 6β-hydroxylation activity by CYP3A4 were determined as previously described.

Standard incubation mixtures of 0.5 ml contained microsomal protein (0.1—0.5 mg), 0.1 M potassium phosphate buffer (pH 7.4), 0.1 mM EDTA, NADPH-generating system (2.5 mM β-NADP, 25 mM glucose-6-phosphate, 2 units of glucose-6-phosphate dehydrogenase, and 10 mM magnesium chloride), and substrates with or without probucol. Probucol was dissolved in dimethyl sulfoxide and added to incubations in a volume of 5 μl. Substrates were dissolved in the following solvents: 7-ethoxyresorufin and 7-benzoxylresorufin in dimethyl sulfoxide; coumarin and bufuralol in ethanol; tolbutamide, S-mephenytoin, and testosterone in methanol; and chlorozoxazone in 1% (w/v) aqueous sodium carbonate. They were added to incubations in a volume of 5 μl. Product formation was determined using HPLC and fluorescence spectrophotometer. Enzyme incubations and metabolite analysis were carried out in duplicate.

Assay methods were validated in this study. Calibration curves for resorufin, 7-hydroxycoumarine, 4-hydroxytolbutamide, 4-hydroxymephenytoin, 1'-hydroxybufuralol, 6-hydrochlorzoxazone, and 6β-hydroxytestosterone were established with respective calibration ranges of 0.2—200 nM (γ = 0.9996), 0.025—5 μM (γ = 1.0000), 0.05—10 μM (γ = 0.9998), 0.025—5 μM (γ = 0.9996), 0.25—100 μM (γ = 0.9994), and 0.03—30 μM (γ = 0.9999).

The substrate concentrations used to estimate the kinetic parameters for each assay were 7-ethoxyresorufin 0.01—10 μM, coumarin 0.1—100 μM, 7-benzoxylresorufin 0.03—10 μM, tolbutamide 25—1000 μM, S-mephenytoin 5—500 μM, bufuralol 5—200 μM, chlorozoxazone 10—400 μM, and testosterone 10—250 μM. For the determination of the residual activity in the presence of probucol (30—300 μM), the concentrations of substrates were 7-ethoxyresorufin 0.5 μM, coumarin 2 μM, 7-benzoxylresorufin 1 μM, tolbutamide 100 μM, S-mephenytoin 100 μM, bufuralol 20 μM, chlorozoxazone 100 μM, and testosterone 100 μM. Selective CYP inhibitors were used in this study to validate that the assays were working properly. 7,8-Benzoflavone, furafylline, orphenadrine, quercetin, sulfaphenazole, tranilcyromine, quinidine, diethylthiocarbamate, and ketoconazole, which are inhibitors of CYP1A1, 1A2, 2B6, 2C8, 2C9, 2C19, 2D6, 2E1, and 3A4 activities, respectively, inhibited the respective enzyme activities (data not shown). Diethylthiocarbamate is also known to be a specific inhibitor of CYP2A6 and the present study confirmed the potent inhibitory capability of this compound on CYP2A6-mediated metabolism (data not shown).

**Data Analysis**

Apparent Kₘ and Vₘₐₓ values for the formation of metabolites were calculated using nonlinear regression analysis on the computer program WinNonlin Standard (Version 2.1, Scientific Consulting, Inc., Apex, NC, U.S.A.).

**RESULTS AND DISCUSSION**

Multiple-drug therapy is a common therapeutic practice, particularly for patients with various diseases. Whenever two or more drugs are administered concurrently, there is the possibility of drug interactions. Many drug interactions are clinically caused by inhibition of drug-metabolizing enzymes, CYPs, leading to decreased metabolic clearance and increased exposure to the inhibited drug. The inhibition of CYP enzymes should thus be examined to assess the potential for drug interactions.

The current study examines the in vitro ability of probucol to inhibit the metabolism of substrates for CYP1A1/2, CYP2A6, CYP2B6, CYP2C8/9, CYP2C19, CYP2D6, CYP2E1, and CYP3A4. The results showed that probucol had neither stimulatory nor inhibitory effects up to a concentration of 300 μM on any of the above CYP-catalyzed reactions (Table 1).

After an oral dose of 1000 mg in healthy volunteers, the peak concentration range of probucol was 8.40 to 16.9 μg/ml (16.3 to 32.7 μM). Moreover, the mean plasma concentration of probucol ranged from 18.2 to 39.2 μg/ml (from 35.2 to 75.8 μM) depending on the duration of probucol therapy after repetitive oral administration of probucol 1000 mg daily for periods of 1 to 12 months. To extrapolate in vitro inhibition data to in vivo situations, free concentrations of drug and inhibitor at the site of metabolism are important. However, this factor is difficult to evaluate in vitro. Often, the in vivo and/or in vitro unbound plasma concentration of a drug and/or inhibitor is used to approximate concentrations of both at an enzyme site. In the whole blood, about 90% of probucol was distributed in the plasma and more than 80% of the total probucol was bound to lipoproteins in the plasma. The free (unbound) concentration of probucol may thus be approximately 0.2 times the total concentration in plasma. The lower plasma level of probucol compared with the highest tested concentration (300 μM) and high plasma protein binding of probucol suggest that probucol might not affect the pharmacokinetics and metabolism of drugs metabolized by CYP1A1/2, 2A6, 2B6, 2C8/9, 2C19, 2D6, 2E1, or 3A4, even if intracellular binding or accumulation of probucol in hepatocytes occurs. There are no clinical reports that probucol causes drug-drug interactions with other CYP-metabolized drugs.

In conclusion, the results of the in vitro experiments performed with human liver microsomes indicate that probucol has neither stimulatory nor inhibitory effects on CYP activities and that this compound, at the expected therapeutic con-
centrations, would not be predicted to cause clinically significant interactions with other CYP-metabolized drugs.

REFERENCES