ROLE OF CYP2C19 IN STEREOSELECTIVE HYDROXYLATION OF MEPHOBARBITAL BY HUMAN LIVER MICROSOMES

KAORU KOBAYASHI,1 MARI KOGO, MASAYOSHI TANI, NORIAKI SHIMADA, TAKASHI ISHZAKI, SATOSHI NUMAZAWA, TAKEMI YOSHIDA, TOSHINORI YAMAMOTO, YUKIO KUROIWA, AND KAN CHIBA

ABSTRACT:

The 4-hydroxylation of mephobarbital enantiomers was investigated by using human liver microsomes from the extensive metabolizers (EM) and poor metabolizers of CYP2C19. The 4-hydroxylase activity of R-mephobarbital in the EM microsomes was >10 times higher than that of S-mephobarbital. In the poor metabolizer microsomes, the 4-hydroxylase activity of R-mephobarbital was much lower than that in the EM microsomes, and the ratio of 4-hydroxylation activity of R-mephobarbital to that of S-mephobarbital was also lower than that in the EM microsomes. Moreover, the 4-hydroxylase activity of R-mephobarbital showed a high correlation \( r = 0.985, p < 0.001 \) with the 4'-hydroxylase activity of S-mephenytoin in a panel of nine human liver microsomes. Anti-CYP2C antibody inhibited R-mephobarbital 4-hydroxylase activity by 85% of the control activity. R-Mephobarbital competitively inhibited S-mephenytoin 4'-hydroxylase activity \( (K_i = 34 \mu M) \), while S-mephenytoin inhibited R-mephobarbital 4-hydroxylase activity \( (K_i = 103 \mu M) \). Among the seven cDNA-expressed CYPs studied, only CYP2C19 catalyzed R-mephobarbital 4-hydroxylation. These findings suggest that the 4-hydroxylation of mephobarbital catalyzed by CYP2C19 is preferential for R-enantiomer in human liver microsomes.

Mephobarbital has been used as an anticonvulsant since 1932. As shown in Fig. 1, this drug is metabolized by N-demethylation to phenobarbital and by 4-hydroxylation to 4-hydroxymephobarbital, which undergoes further glucuronidation (Butler et al., 1952; Hooper et al., 1981). Mephobarbital is a chiral compound that is commercially available as a racemate of mephobarbital, whereas only 7% of S-mephobarbital was converted to the corresponding hydroxy metabolite (Lim and Hooper, 1989). Kupfer and Branch (1985) reported that the urinary recovery of 4-hydroxymephobarbital after administration of mephobarbital was negligible in poor metabolizers (PM2) of S-mephenytoin. These authors also measured the urinary recovery of 4-hydroxymephobarbital after administration of R- or S-mephobarbital to an extensive metabolizer (EM) of S-mephenytoin and showed that 4-hydroxymephobarbital was detected in urine when R-mephobarbital, but not S-mephobarbital, was administered. These findings suggest that 4-hydroxymephobarbital had exclusively been derived from R-mephobarbital and that its formation from R-mephobarbital cosegregates with the genetically determined activity of S-mephenytoin 4'-hydroxylase, designated as cytochrome P450 2C19 (CYP2C19).

However, to our knowledge, no in vitro data on the hydroxylation of mephobarbital enantiomers have been published. The purpose of this study was to clarify the stereoselective hydroxylation of mephobarbital in relation to S-mephenytoin 4'-hydroxylase in human liver microsomes and cDNA-expressed CYP isoforms.

Materials and Methods

Chemicals. Racemic mephobarbital was supplied by Yoshitomi Pharmaceutical Co. (Osaka, Japan), and phenobarbital was supplied by Dainippon Pharmaceutical Co. (Osaka, Japan). 4-Hydroxyphenobarbital was purchased from Aldrich Japan (Tokyo, Japan), and cyclohexane was purchased from Tokyo Chemical Industry Co. (Tokyo, Japan). R- and S-mephobarbital were separated from the racemic mixture using a HPLC system equipped with a Chiralcel CA-1 column (20 × 250 mm; Daisel Chemical Co., Tokyo, Japan). Ethanol was eluted at flow rate of 4.5 ml/min. The eluate was monitored at a wavelength of 254 nm.

1 Present address: Laboratory of Biochemical Pharmacology and Toxicology, Faculty of Pharmaceutical Sciences, Chiba University, Chiba 263-8522, Japan.

2 Abbreviations used are: PM, poor metabolizer; CYP, cytochrome P450; EM, extensive metabolizer; HPLC, high-performance liquid chromatography.
wavelength of 280 nm. The column temperature was maintained at 40°C. 4-Hydroxymephenobarbital was prepared from 4-hydroxyphenobarbital via N-methylation using dimethyl sulfate as described by Hiers and Hager (1961). Racemic mephenytoin and 4′-hydroxymephenytoin were kindly donated by Dr. Küpfer (University of Berne, Berne, Switzerland). S- and R-mephénytoin were separated from the racemic mixture of mephenytoin by a Chira[el OJ column (10 μm, 4.6 × 250 mm; Daisel Chemical Co.) as reported by Yasumori et al. (1990). NADP⁺ and glucose 6-phosphate were purchased from Oriental Yeast Co. (Tokyo, Japan). Glucose-6-phosphate dehydrogenase was purchased from Boehringer Mannheim GmbH (Mannheim, Germany). HPLC-grade acetonitrile and other reagents of analytical grade were purchased from Wako Pure Chemical Industries (Osaka, Japan).

Enzymes. Nine samples of human liver microsomes were obtained from Japanese patients undergoing partial hepatectomy for treatment of metastatic liver tumors at the Division of Surgery, International Medical Center of Japan (Tokyo, Japan) and were prepared as reported previously (Chiba et al., 1993). Among the nine microsomal samples used in the present study, two samples were considered to be derived from the CYP2C19-related PM patients, since CYP2C19*2 and CYP2C19*3, which are the most frequently seen CYP2C19 alleles in the Japanese population (Kubota et al. 1996). Genotyping procedures were considered to be derived from the CYP2C19-related PM patients, since CYP2C19*2 and CYP2C19*3 alleles were performed by a polymerase chain reaction-restriction fragment length polymorphism method as described by de Morais et al. (1994a,b) with minor modifications (Kubota et al., 1996). The characteristics of the nine human livers are shown in Table 1.

Microsomes prepared from human B-lymphoblastoid cells expressing human CYP1A2 (lot 29), CYP2A6 (lot 17), CYP2B6 (lot 20), CYP2C19 (lot 1), CYP2D6 (lot 38), CYP2E1 (lot 18), and CYP3A4 (lot 4) were obtained from GENTEST Corp. (Woburn, MA). cDNA-expressed CYP2E1 and CYP3A4 were coexpressed with NADPH-CYP reductase in human B-lymphoblastoid cells.

Assay with Human Liver Microsomes. The primary incubation medium contained 25 or 50 μg of microsomal protein, 0.1 mM EDTA, 100 mM potassium phosphate buffer (pH 7.4), an NADPH-generating system (0.5 M NADP⁺, 2.0 mM glucose 6-phosphate, 1 U/mL of glucose-6-phosphate dehydrogenase, and 4 mM MgCl₂), and R-mephénytoin or S-mephénytoin, in a final volume of 250 μL. The mixture was incubated at 37°C for 60 min. After the reaction was stopped by the addition of 100 μL of cold acetonitrile, 50 μL of cyclobarbital (1.25 μg/mL in methanol) was added to the samples as an internal standard. The mixture was centrifuged at 10,000 g for 5 min, and 100 μL of the supernatant was analyzed under HPLC conditions as described below.

HPLC Conditions. The determination of 4-hydroxymepobarbital was carried out using an Hitachi HPLC system (Tokyo, Japan) consisting of an L-6000 pump, an L-4000 UV detector, an AS-2000 autosampler, a D-2500 integrator, and a CAPCELL PAK C18 UG120 column (5 μm, 4.6 × 250 mm; Shimadzu, Tokyo, Japan). The mobile phase consisted of 50 mM potassium phosphate buffer (pH 5.0) and acetonitrile at the ratio of 75:25 (v/v) and was delivered at a flow rate of 1 mL/min. The eluate was monitored at a wavelength of 204 nm. The column temperature was maintained at 30°C. Under these chromatographic conditions, 4-hydroxymepobarbital and cyclobarbital were eluted at 9.3 and 14.3 min, respectively. The detection limit of 4-hydroxymepobarbital was 2 pmol in an incubation mixture of 250 μL. 4-Hydroxymepobarbital was quantified by comparison with the standard curves, by using the peak-height ratio method. Intra-assay (n = 6) coefficients of variation were less than 7%.

Correlation Studies. The 4-hydroxylation activities of R- and S-mephénytoin were compared with the S-mephénytoin 4′-hydroxylation activity using microsomes obtained from nine human livers. R- and S-mephénytoin were used at a concentration of 100 mM. Assays were performed in duplicate on the same day, with the same set of microsomal preparations. S-Mephénytoin (100 μM) was incubated with 0.2 mg/mL microsomal protein for 60 min. Determination of 4′-hydroxymepobarbital was carried out as reported previously (Chiba et al., 1993).

Immuno-inhibition Studies. The immuno-inhibition of 4-hydroxylase activity of R-mephénytoin was examined by preincubating the human liver microsomal samples (25 μg of human liver microsomes) with various amounts (0–2 mg of IgG/mg of microsomal protein) of preimmune IgG or anti-CYP2C IgG in 0.1 M potassium phosphate buffer (pH 7.4) for 30 min at room temperature. R-Mephénytoin (100 μM) and the other reagents of the incubation medium were added, and the reaction was carried out as described above. Anti-CYP2C antibody used in the present study inhibited S-mephénytoin 4′-hydroxylation activity (CYP2C19) and tolbutamide hydroxylation activity (CYP2C9) by more than 90%, whereas it did not inhibit testosterone 6β-hydroxylation activity (CYP3A) in human liver microsomes (Kobayashi et al., 1997).

Chemical Inhibition Studies. The inhibition of R-mephénytoin 4′-hydroxylation activity by S-mephénytoin was studied at the substrate concentrations of 50, 100, and 200 μM in the presence of five concentrations of S-mephénytoin from 0 to 200 μM. The inhibition of S-mephénytoin 4′-hydroxylation activity by R-mephénytoin was studied at the substrate concentrations of 50, 100, and 200 μM in the presence of four concentrations of R-mephénytoin from 0 to 200 μM. Inhibition patterns were determined by a visual inspection of the double reciprocal plots of S-mephénytoin concentration versus the velocity of the reaction. Apparent Kᵢ values were determined by a visual inspection of the double reciprocal plots of S-mephénytoin concentration versus the velocity of the reaction. Apparent Kᵢ (inhibition constant) values were determined by an unweighted linear regression analysis using an equation consistent with the competitive inhibition:

$$K_{i,app} = K_i + K_m \cdot I/K_s$$
Substrates (R- and S-mephobarbital) at 100 μM were incubated at 37°C for 60 min with 0.2 mg/ml of human liver microsomes. Each of the columns represents the mean of duplicate experiments. ■, R-enantiomer; □, S-enantiomer.

Results

4-Hydroxylase Activities of R- and S-Mephobarbital in Human Liver Microsomes. The 4-hydroxylase activities of R- and S-mephobarbital in nine human liver microsomes, including the two CYP2C19-related PM microsomes, are shown in Fig. 2. The 4-hydroxylase activity of R-mephobarbital in the nine human liver microsomes was 3 to 20 times higher than that of S-mephobarbital-related PM microsomes. Microsomes from human B-lymphoblastoid cells expressing human CYP1A2, CYP2A6, CYP2B6, CYP2C19, CYP2D6, CYP2E1, and CYP3A4 were used. The reactions were carried out as described for the human liver microsomal study. To examine the role of individual CYP isoforms involved in 4-hydroxylation of R- or S-mephobarbital, each of the cDNA-expressed CYPs (0.5 mg/ml of protein concentration) described above was incubated with R- or S-mephobarbital (each at 100 μM) for 2 h at 37°C, according to the procedure recommended by the supplier.

Kinetic Studies. The 4-hydroxylase activities of R-mephobarbital were determined at substrate concentrations ranging from 40 to 300 μM. All reactions were performed within a linear range with respect to protein concentration and incubation time (i.e., 0.2 mg/ml microsomal protein and 60-min incubation time). The kinetics parameters (K_m and V_max) were estimated by graphic analysis of Eadie-Hofstee plots. The values were subsequently used as initial estimates for nonlinear least-squares regression analysis. The intrinsic clearance values were calculated as V_max/K_m.

Assay with cDNA-Expressed CYPs. Microsomes from human B-lymphoblastoid cells expressing human CYP1A2, CYP2A6, CYP2B6, CYP2C19, CYP2D6, CYP2E1, and CYP3A4 were used. The reactions were carried out as described for the human liver microsomal study. To examine the role of individual CYP isoforms involved in 4-hydroxylation of R- or S-mephobarbital, each of the cDNA-expressed CYPs (0.5 mg/ml of protein concentration) described above was incubated with R- or S-mephobarbital (each at 100 μM) for 2 h at 37°C, according to the procedure recommended by the supplier.

Correlation of 4-hydroxylase activities of R- and S-mephobarbital with S-mephenytoin 4'-hydroxylase activity in nine human liver microsomes. Substrates at 100 μM were incubated at 37°C for 60 min with 0.2 mg/ml of human liver microsomes. Each of the data points represents the mean of duplicate experiments. The correlation coefficients (r) were calculated by the least-squares regression method. All microsomal samples listed in Table 1 were used in this study.

4-Hydroxylation of R-mephobarbital in human liver microsomes. Human liver microsomes (25 μg of microsomal protein, HL-7) were preincubated with anti-CYP2C IgG (●) or preimmune IgG (○) for 30 min at room temperature before incubation with 100 μM R-mephobarbital. The control activity in the absence of antibodies was 0.75 nmol/mg/h. Each of the data points represents the mean of duplicate experiments.

Human liver microsomes (25 μg of microsomal protein, HL-7) were preincubated with anti-CYP2C IgG (●) or preimmune IgG (○) for 30 min at room temperature before incubation with 100 μM R-mephobarbital. The control activity in the absence of antibodies was 0.75 nmol/mg/h. Each of the data points represents the mean of duplicate experiments.
between the EM and PM microsomes. The Michaelis-Menten kinetics parameters derived from the one-enzyme kinetics approach in the EM (HL-5, -6, -7, and -23) and PM microsomes (HL-8 and -22) of CYP2C19 are listed in Table 2. The apparent $K_m$ values for the 4-hydroxylation were higher in the PM microsomes than in the EM microsomes (457 and 273 $\mu$M versus 65 ± 21 $\mu$M), while no difference was found between the EM and PM microsomes in $V_{max}$ values, resulting in the observation that the intrinsic clearance values were 3 to 9 times lower in the PM microsomes than in the EM microsomes (2.3 and 5.3 versus 20.6 ± 5.4 $\mu$l/mg/h).

Activity in cDNA-Expressed CYPs. Microsomes from human B-lymphoblastoid cells expressing each of the seven human CYP isoforms were examined in terms of the ability of their CYP proteins to catalyze the 4-hydroxylation of $R$- or $S$-mephenytoin. The formation of hydroxymephenytoin from $R$-mephenytoin was catalyzed by only CYP2C19 (1413 pmol/mg/2 h). For the formation of hydroxymephenytoin from $S$-mephenytoin, all cDNA-expressed CYPs screened showed a negligible activity (< 16 pmol/mg/2 h).

Discussion

The pharmacokinetics and metabolism of mephenytoin are recognized to be stereoselective. Kupfer and Branch (1985) reported that 33% of hydroxymephenytoin was recovered in urine after administration of $R$-mephenytoin, whereas the urinary excretion was negligible after administration of $S$-mephenytoin. After a single-dose administration of racemic mephenytoin, the oral clearance of $R$-mephenytoin was much greater than that of $S$-mephenytoin in six adult male volunteers (Lim and Hooper, 1989). $R$-Mephenytoin was extensively hydroxylated, and about 50% of $R$-mephenytoin was recovered in urine as $R$-hydroxymephenytoin, whereas the elimination of $S$-mephenytoin was extremely slow, and only 7% of $S$-mephenytoin was converted to $S$-hydroxymephenytoin.

In this study using human liver microsomes, the mean 4-hydroxylase activity of $R$-enantiomer was 3 to 20 times higher than that of $S$-enantiomer in human liver microsomes (Fig. 2). In the EM microsomes, the mean $R/S$ ratio was 13.5, suggesting that the 4-hydroxylation of mephenytoin was preferential for $R$-enantiomer. On the other hand, the 4-hydroxylase activities of $R$-mephenytoin in the two PM microsomes were much lower than those in the EM microsomes (Fig. 2), and the $R/S$ ratios in the two PM microsomes (i.e., 3.6 and 6.1) were also lower than the mean $R/S$ ratio in the EM microsomes. This result suggests that the 4-hydroxylation of mephenytoin is less stereoselective in the PM microsomes. These findings obtained from the present in vitro study are in good agreement with the in vivo observation reported by Kupfer and Branch (1985).

Since the current in vitro and previous in vivo studies (Kupfer and Branch, 1985; Lim and Hooper, 1989) suggest that the stereoselective metabolism of mephenytoin is mainly attributable to the preferential hydroxylation of $R$-mephenytoin, we investigated which CYP isoform(s) is involved in the 4-hydroxylation of $R$-mephenytoin in human liver microsomes. The following results were obtained. First, a significant correlation existed between the activity of $R$-mephenytoin 4-hydroxylation and that of $S$-mephenytoin 4'-hydroxylation in the nine human liver microsomes (Fig. 3). Second, the $R$-mephenytoin 4-hydroxylation was inhibited almost completely by the addition of anti-CYP2C antibody (Fig. 4). Third, there was mutual competitive inhibition of $R$-mephenytoin 4-hydroxylation and $S$-mephenytoin 4'-hydroxylation. Fourth, the EM microsomes had a greater mean intrinsic clearance value for $R$-mephenytoin 4-hydroxylation compared with the respective intrinsic clearance values of the two PM microsomes (Table 2). Fifth, only cDNA-expressed CYP2C19 catalyzed $R$-mephenytoin 4-hydroxylation, and the other cDNA-expressed CYP isoforms screened showed a negligible activity. These data clearly indicated that CYP2C19 is mainly responsible for the 4-hydroxylation of $R$-mephenytoin in human liver microsomes.

On the other hand, the 4-hydroxylase activity of $S$-mephenytoin was not substantially discernible in any of the cDNA-expressed CYPs examined. Therefore, $S$-mephenytoin is considered to be barely 4-hydroxylated in human liver microsomes, being consistent with an in vivo observation that the excretion of hydroxymephenytoin into urine was negligible in an EM after the administration of $S$-mephenytoin (Kupfer and Branch, 1985). Previous in vivo studies suggested that most of the circulating phenobarbital seemed to be derived from $S$-mephenytoin (Kupfer and Branch, 1985; Lim and Hooper, 1989). In addition, our in vitro data indicated that $S$-mephenytoin was mainly N-demethylated by CYP2B6 (Kobayashi et al., 1999). Taken together, a main metabolic route of $S$-mephenytoin in vivo and in vitro appears to be the N-demethylation, but not 4-hydroxylation.

The 4'-hydroxylation of mephenytoin by CYP2C19 is known to be stereospecific for $S$-enantiomer (Goldstein et al., 1994). Our observation that $R$-mephenytoin is preferentially 4-hydroxylated by CYP2C19 as similar to the 4'-hydroxylation of $S$-mephenytoin requires an assumptive discussion. The preferential hydroxylation of $R$-mephenytoin and $S$-mephenytoin by CYP2C19 may be explained as follows. When the ureido groups of $S$-mephenytoin and $R$-mephenytoin are defined as being located in the horizontal position in a
three-dimensional model, the 4-position of the phenyl ring (i.e., oxidized site) is located under the ureido surface. CYP2C19 may recognize the carbonyl moiety and phenyl ring of S-mephobarbital and R-mephobarbital precisely in the substrate binding site of CYP2C19. This assumptive explanation appears to be consistent with the proposition by Yasumori et al. (1999).

The present study suggested that R-mephobarbital was extensively 4-hydroxylated via CYP2C19 in human liver microsomes. This finding is consistent with the in vivo observation that the extensive 4-hydroxylation of R-mephobarbital resulted in rapid elimination of this enantiomer in the EM of CYP2C19 (Lim and Hooper, 1989; Hooper and Qing, 1990). At least in the EM of CYP2C19, 4-hydroxylation of mephobarbital seems to be important in the overall metabolism of this drug. In contrast, the PM of CYP2C19 is deficient in R-mephobarbital 4-hydroxylation. In PM of CYP2C19, R-mephobarbital would be anticipated to exhibit a low clearance similar to that of S-mephobarbital. Mephobarbital possesses anticonvulsant property in its own right (Craig and Shideman, 1971), although it is frequently regarded as a prodrug for phenobarbital. Therefore, an exaggerated reaction to this drug might be caused in the PM of CYP2C19.

In closing, to our knowledge, this is the first in vitro study on the hydroxylation of mephobarbital enantiomers using human liver microsomes and cDNA-expressed CYPs, indicating that 4-hydroxylation of mephobarbital more dominantly occur for R-enantiomer than for S-enantiomer and that the 4-hydroxylation of R-mephobarbital is primarily catalyzed by CYP2C19. Because the 4-hydroxylation of R-mephobarbital is a CYP2C19-specific reaction as observed for S-mephenytoin 4′-hydroxylation, we are tempted to propose that R-mephobarbital is an alternative phenotyping probe for assessing CYP2C19 activity in humans.

References


