CYTOCHROME P450 2C9 CATALYZES INDOMETHACIN O-DEMETHYLATION IN HUMAN LIVER MICROSOMES

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ABSTRACT:
Indomethacin is a widely used nonsteroidal anti-inflammatory drug. We studied the human cytochrome P450 (CYP) isoform responsible for indomethacin O-demethylation, the major metabolic pathway for indomethacin. For indomethacin O-demethylase activities, the $K_M$ value was 34.6 ± 5.4 μM and the $V_{max}$ value was 14.1 ± 3.9 pmol/mg/min in human liver microsomes (N = 4). Indomethacin O-demethylase activity in human liver microsomes was competitively inhibited by sulfaphenazole, (S)-warfarin, and tolbutamide and was not affected by α-naphthoflavone, (S)-mephenytoin, or erythromycin. Indomethacin O-demethylase activities in microsomes from nine human livers were significantly correlated with tolbutamide hydroxylase activities ($r = 0.750, p < 0.05$) and not with (S)-mephenytoin 4'-hydroxylase activities. When the capacity for indomethacin O-demethylation in microsomes of B lymphoblastoid cells expressing human CYPs was investigated at an indomethacin concentration of 5 μM, cDNA-expressed CYP2C9 exhibited 6-fold greater activity than did CYP2C19. At an indomethacin concentration of 50 μM, cDNA-expressed CYP1A2 and CYP2D6 also exhibited slight activities. The $K_M$ values were 9.9 ± 1.2 and 117.1 ± 13.8 μM and the $V_{max}$ values were 0.33 ± 0.05 and 0.24 ± 0.04 pmol/min/pmol CYP in microsomes with cDNA-expressed CYP2C9 and CYP2C19, respectively (N = 4). Considering the 16-fold higher intrinsic clearance of CYP2C9, compared with that of CYP2C19, and these expression levels in human livers, the contribution of CYP2C9 to indomethacin O-demethylation was considered to be negligible. Indomethacin appears to be O-demethylated exclusively by CYP2C9 in humans.

CYPs are a superfamily of hemoproteins that are organized into families and subfamilies based on similarities in their amino acid sequences. At least 10 CYP families have been described in mammals, and >20 human CYP gene products have been characterized (Nelson et al., 1996). CYP is responsible for the oxidative metabolism of many drugs, environmental chemicals, and endogenous compounds (Wrighton and Stevens, 1992). Xenobiotic-metabolizing CYP isoforms exhibit characteristic, but frequently overlapping, patterns of substrate specificities (Gonzalez, 1992). Prediction of drug interactions and other environmental and genetic factors that influence the metabolism of any drug requires identification of the CYP isoform(s) responsible for its metabolism. In recent years, a number of approaches have been developed for identification of the human CYP isoforms involved in the metabolism of particular xenobiotics in vitro (Birkett et al., 1993; Miners et al., 1994).

Most NSAIDs are predominantly eliminated in humans by hepatic biotransformation, and a number of major compounds in this therapeutically class undergo extensive oxidation, with wide interindividual variability. It has been demonstrated that CYP2C9, which is known to be a tolbutamide hydroxylase (Veronese et al., 1991), catalyzes oxidative metabolism of NSAIDs such as diclofenac (Leemann et al., 1993b), ibuprofen (Leemann et al., 1994), mfenamic acid (Bonnabry et al., 1994), and piroxicam (Kondo et al., 1992). Another NSAID, indomethacin, is also used widely throughout the world. The extensive metabolism of indomethacin into O-desmethylindomethacin, N-deschlorobenzoylindomethacin, and O-desmethyl-N-deschlorobenzoylindomethacin has been shown (Harman et al., 1964) (fig. 1). These metabolites are devoid of anti-inflammatory activity (Duggan et al., 1972). In humans, O-desmethylindomethacin formation is critical to the elimination of indomethacin and represents 40–55% of total drug eliminated in the urine (Duggan et al., 1972). It has been reported that O-desmethylindomethacin is formed by CYP in humans (Duggan et al., 1972); however, which isoforms catalyze the formation is not clear. The objective of this study was to identify the principal CYP isoform(s) involved in indomethacin O-demethylation in humans, to clarify the possible drug interactions of indomethacin.

Materials and Methods

Chemicals. Indomethacin was purchased from Sigma Chemical Co. (St. Louis, MO). O-Desmethylinidomethacin was kindly provided by Merck Sharp and Dohme Research Laboratories (Rahway, NJ). (S)+(-)-Mephenytoin, (±)-4'-hydroxymephenytoin, hydroxytolbutamide, sulfaphenazole, and (S)(−)-warfarin were purchased from Ultrafine Chemicals (Manchester, UK). NADPH, glucose-6-phosphate, and glucose-6-phosphate dehydrogenase were from Oriental Yeast (Tokyo, Japan). Other chemicals were of the highest grade commercially available. Microsomes from human B lymphoblastoid cell lines...
and centrifuged at 3000 rpm for 10 min, to separate the aqueous and organic
centrifugation, the reaction mixtures were extracted with 4 ml of ethyl acetate
(2.5 ng) was added as an internal standard. After removal of protein by

The eluent was monitored at 260 nm using a UV-970 intelligent UV/visible

for determination of kinetic parameters. The values were used to estimate apparent kinetic parameters by linear least-squares regression analysis.

cDNA-Expressed CYP Incubations. In this experiment, microsomes from human B lymphoblastoid cell lines expressing CYP1A1, CYP1A2, CYP2A6, CYP2B6, CYP2C8, CYP2C9-Arg Arg, CYP2C9-Cys Cys Arg (this major type is referred to as CYP2C9 in this article), CYP2C9-Cys Cys Arg, CYP2C19, CYP2D6, CYP2E1, or CYP3A4 were used. Control microsomes were from human B lymphoblastoid cells that had not been transfected. CYP2A6, CYP2C8, CYP2C9-Arg Arg, CYP2C9-Cys Cys Arg, CYP2D6, CYP2E1, and CYP3A4 were coexpressed (from cDNA) with NADPH-CYP reductase in microsomes. Other isozymes were not coexpressed. Production of metabolites by these microsomes was approximately linear for times up to at least 2 hr, using each specific substrate. The incubation mixture was of the same composition as described above, except with a microsomal protein concentration of 1.0 mg/ml. The indomethacin concentration used was 5 or 50 μM. Unless specified, the mixture was incubated at 37°C for 60 min after a 2-min preincubation.

Inhibition Analyses. The effects of CYP inhibitors on indomethacin O-
demethylase activities in human liver microsomes were investigated. The inhibitors studied were α-naphthoflavone (Butler et al., 1989), sulfaphenazole (Baldwin et al., 1995), (S)-warfarin (Kaminsky and Zhang, 1997), tolbutamide (Veronese et al., 1991), (S)-mephénytoin (Chiba et al., 1993), and erythromycin (Watts et al., 1985). For determination of IC50 values, the indomethacin concentration was 30 μM. The range of inhibitor concentrations was 1–100 μM. For determination of Ks values, indomethacin concentrations were 30, 60, and 100 μM. The range of inhibitor concentrations was 3–10 μM, 50–500 μM, and 0.1–1.0 mM for sulfaphenazole, (S)-warfarin, and tolbutamide, respectively. The incubation mixture, including chemical inhibitors, was preincubated for 2 min before the reaction was initiated by the addition of the NADPH-generating system. The assays were performed as described above.

Tolbutamide Hydroxylation. The activities in human liver microsomes were determined as described previously (Relling et al., 1990), with slight modifications. The incubation mixture (0.5-ml total volume) contained 0.1 M potassium phosphate buffer (pH 7.4), the NADPH-generating system, 0.25 mg/ml microsomal protein, and 1 mM tolbutamide as substrate. The reaction was initiated by the addition of the NADPH-generating system, after a 2-min preincubation at 37°C. After incubation for 30 min at 37°C, the reaction was terminated by addition of 100 μl of 1 N hydrochloric acid, and phenacetin (20 ng) was added as an internal standard. After removal of protein by centrifugation, the reaction mixtures were extracted with 3 ml of chloroform and centrifuged at 3000 rpm for 10 min, to separate the aqueous and organic fractions. The organic fraction was evaporated under a gentle stream of nitrogen at 40°C. The residue was redissolved in 50 μl of methanol, and then 20-μl portions of the samples were subjected to HPLC. The HPLC apparatus was the same as described above, without the Uni-2s baseline adjuster. The analytical column used was a Mightysil RP-18 column (4.6 mm i.d. × 250 mm, 5 μm; Kanto Chemical, Tokyo, Japan). The eluent was monitored at 260 nm using a UV-970 intelligent UV/visible detector (Jasco) with a Uni-2s baseline adjuster (Union, Gunma, Japan). The mobile phase was 40% acetonitrile containing 0.6% acetic acid, the flow rate was 1.0 ml/min, and the column temperature was 35°C.

(S)-Mephénytoin Hydroxylation. The activities in human liver microsomes were determined as described previously (Meier et al., 1985), with slight modifications. The incubation mixture (0.25-ml total volume) contained 0.1 M potassium phosphate buffer (pH 7.4), the NADPH-generating system, 0.5 mg/ml microsomal protein, and 200 μM (S)-mephénytoin as substrate. The reaction was initiated by the addition of the NADPH-generating system, after a 2-min preincubation at 37°C. After incubation for 60 min at 37°C, the reaction was terminated by addition of 100 μl of 1 N hydrochloric acid, and phenacetin (20 ng) was added as an internal standard. After removal of protein by centrifugation, the reaction mixtures were extracted with 3 ml of chloroform and centrifuged at 3000 rpm for 10 min, to separate the aqueous and organic fractions. The organic fraction was evaporated under a gentle stream of nitrogen at 40°C. The residue was redissolved in 50 μl of methanol, and then 20-μl portions of the samples were subjected to HPLC. The HPLC apparatus was the same as described above, without the Uni-2s baseline adjuster. The analytical column used was a Mightysil RP-18 column (4.6 mm i.d. × 250 mm, 5 μm), and the hydroxytolbutamide formed was monitored at 236 nm. The mobile phase was 30% acetonitrile containing 0.5% acetic acid, the flow rate was 1.0 ml/min, and the column temperature was 35°C.

-expressed CYP2C9. Eadie-Hofstee plots

expressing human CYP and control microsomes that had not been transfected were obtained from Gentest (Woburn, MA).

Tissue Samples and Preparation of Microsomes. Human liver samples were obtained from autopsies. The use of human livers for this study had been approved by the Institutional Committee of Tokyo Medical Examiner’s Office. Liver tissues were rapidly frozen in liquid nitrogen immediately after excision and were stored at −80°C. Microsomes from the human livers were prepared as described previously (Kamataki and Kitagawa, 1974) and were stored at −80°C until used. Protein concentrations were measured according to the method of Lowry et al. (1951). CYP contents were estimated by the method of Johannesen and Depierre (1978).

Indomethacin O-Demethylation. The formation of O-demethylindomethacin in human liver microsomes was determined by HPLC. A typical incubation mixture consisted of 50 mM potassium phosphate buffer (pH 7.4), an NADPH-generating system (0.5 mM NADP+, 5 mM glucose-6-phosphate, 5 mM MgCl2, and 1 unit/ml glucose-6-phosphate dehydrogenase), indomethacin dissolved in dimethylsulfoxide (final concentration of dimethylsulfoxide, <1%), and 0.5 mg/ml microsomal protein, in a final volume of 250 μl. The reaction was initiated by the addition of the NADPH-generating system, after a 2-min preincubation at 37°C. After incubation for 30 min, the reaction was terminated by the addition of 150 μl of ice-cold acetonitrile, and ketoprofen (2.5 ng) was added as an internal standard. After removal of protein by centrifugation, the reaction mixtures were extracted with 4 ml of ethyl acetate and centrifuged at 3000 rpm for 10 min, to separate the aqueous and organic fractions. The organic fraction was evaporated under a gentle stream of nitrogen at 40°C. The residue was redissolved in 30 μl of methanol and diluted to 60 μl with deionized water. A 30-μl portion of the sample was subjected to HPLC. HPLC analyses were performed using a DG-980–50 degasser (Jasco, Tokyo, Japan), a PU-980 intelligent pump (Jasco), an AS-950 intelligent sampler (Jasco), and an 807-IT integrator (Jasco) equipped with a Mightysil RP-18 column (4.6 mm i.d. × 250 mm, 5 μm; Kanto Chemical, Tokyo, Japan). The eluent was monitored at 260 nm using a UV-970 intelligent UV/visible detector (Jasco) with a Uni-2s baseline adjuster (Union, Gunma, Japan). The mobile phase was 40% acetonitrile containing 0.6% acetic acid, the flow rate was 1.0 ml/min, and the column temperature was 35°C.

-Deschlorobenzoylindomethacin and O-desmethyl-N-deschlorobenzoylindomethacin.

FIG. 1. Primary metabolic pathways for indomethacin in humans.
activity and tolbutamide hydroxylase activity or (S)-mephentoin 4'-hydroxylase activity in human liver microsomes were determined by Pearson’s product-moment method. Results are expressed as mean ± SE.

Results

Kinetic Analyses of Indomethacin O-Demethylase Activities in Human Liver Microsomes. Indomethacin O-demethylase activity in human liver microsomes increased linearly with incubation times up to 60 min and with protein concentrations up to 1.0 mg/ml (data not shown). Unless specified, an incubation time of 30 min and a concentration of 0.5 mg/ml microsomal protein were used to ensure initial rate conditions for indomethacin O-demethylation. Eadie-Hofstee plots for indomethacin O-demethylase activities in microsomes from four human livers were linear. Fig. 2 shows a representative plot for indomethacin O-demethylase activity in human liver microsomes. The $K_m$ value was 34.6 ± 5.4 μM and the $V_{max}$ value was 14.1 ± 3.9 pmol/min/mg in the four microsome samples (table 1).

Indomethacin O-Demethylase Activities in Microsomes of B Lymphoblastoid Cells Expressing Human CYPs. The capabilities for O-desmethylindomethacin formation in microsomes of B lymphoblastoid cells expressing human CYPs were determined (fig. 3). At a 5 μM substrate concentration, cDNA-expressed CYP2C9 and CYP2C19 exhibited O-desmethylindomethacin formation (0.252 and 0.042 pmol/min/mg CYP, respectively). At a 50 μM substrate concentration, CYP1A2 (0.006 pmol/min/mg CYP) and CYP2D6 (0.004 pmol/min/mg CYP) also exhibited O-desmethylindomethacin formation, as did CYP2C9 (0.493 pmol/min/mg CYP) and CYP2C19 (0.106 pmol/min/mg CYP). cDNA-expressed CYP2C9 showed the highest indomethacin O-demethylase activity of all isoforms investigated in this study. Incubations with cDNA-expressed CYP1A1, CYP2A6, CYP2B6, CYP2C8, CYP2E1, and CYP3A4 did not produce detectable levels of O-desmethylindomethacin, even at high substrate concentrations.

Inhibition Analyses. The CYP isoform-specific xenobiotic probes were screened for inhibitory effects on indomethacin O-demethylase activities. Fig. 4 shows the mean activities (from duplicate determinations) in the presence of these inhibitors. Sulphaphenazole, (S)-warfarin, and tolbutamide, which are specific substrates for CYP2C9, inhibited the indomethacin O-demethylase activity in human liver microsomes. $IC_{50}$ values were 0.9, 47.1, and 97.7 μM for sulphaphenazole, (S)-warfarin, and tolbutamide, respectively. No effects of the other xenobiotic inhibitors, i.e. α-naphthoflavone (CYP1A2), (S)-mephentoin (CYP2C19), and erythromycin (CYP3A4), on indomethacin O-demethylase activities were observed with inhibitor concentrations up to 100 μM.

Fig. 5 shows Lineweaver-Burk plots of indomethacin O-demethylase activity in human liver microsomes. The $K_i$ values were 6.3, 8.7, and 120.4 μM for sulphaphenazole, (S)-warfarin, and tolbutamide, respectively.

Relationship between Indomethacin O-Demethylase Activities
and Tolbutamide Hydroxylase or (S)-Mephenytoin 4'-Hydroxylase Activities in Human Liver Microsomes. Indomethacin O-demethylase activities in microsomes from nine human livers were determined with a 50 μM substrate concentration. Tolbutamide hydroxylase and (S)-mephenytoin 4'-hydroxylase activities in the human liver microsomes were determined as probes of CYP2C9 and CYP2C19 activities, respectively. As shown in fig. 6, indomethacin O-demethylase activities were significantly correlated with tolbutamide hydroxylase activities \( r = 0.750, p < 0.05 \) but were not correlated with (S)-mephenytoin 4'-hydroxylase activities \( r = 0.250 \).

Kinetic Analyses of Indomethacin O-Demethylase Activities in Microsomes with cDNA-Expressed CYP2C9 or CYP2C19. Kinetic analyses were performed using microsomes expressing CYP2C9 or CYP2C19 cDNA. Eadie-Hofstee plots for indomethacin O-demethylation in these microsomes were linear (data not shown). Fig. 7 shows the kinetic parameters for indomethacin O-demethylation in microsomes from these expression systems. In microsomes expressing CYP2C9 cDNA, the \( K_i \) value was 9.9 ± 1.2 μM and the \( V_{\text{max}} \) value was 0.33 ± 0.05 pmol/min/pmol CYP \( (N = 4) \). In microsomes expressing CYP2C19 cDNA, the \( K_i \) value was 117.1 ± 13.8 μM and the \( V_{\text{max}} \) value was 0.24 ± 0.04 pmol/min/pmol CYP \( (N = 4) \). The \( V_{\text{max}}/K_i \) values for CYP2C9 and CYP2C19 were 36.1 ± 8.2 and 2.2 ± 0.5 nl/min/pmol CYP, respectively. Therefore, the \( V_{\text{max}}/K_i \) value for CYP2C9 was 16-fold higher than that for CYP2C19.

Discussion

Recently, a number of approaches have been developed for the identification of human CYPs responsible for the metabolism of particular drugs. In those studies, NSAIDs such as diclofenac (Lee- mann et al., 1993b), mafenamic acid (Bonnabry et al., 1994), and piroxicam (Kondo et al., 1992) were demonstrated to be metabolized by CYP2C9. Indomethacin (an NSAID) has been used widely; nevertheless, its metabolism has not been studied thoroughly. Indomethacin is metabolized via O-demethylation and N-deacylation. The N-deacylation pathway, i.e. the formation of N-deschlorobenzoylindomethacin from indomethacin, seems not to be dependent on CYPs. Recently, the carboxylesterase that catalyzes the N-deacylation of indomethacin was purified from pig liver (Terashima et al., 1996).

The O-demethylation pathway is critical for the elimination of indomethacin (Duggan et al., 1972). Although O-demethylation of indomethacin has been reported to be catalyzed by CYP in humans (Duggan et al., 1972), the isoform involved in the reaction has not been clarified. In this study, we identified the human CYP isoform involved in indomethacin O-demethylation.

Previously, it was reported that most NSAIDs, including indomethacin, inhibit tolbutamide hydroxylase activity in human liver microsomes (Leemann et al., 1993a). Leemann and colleagues reported that the \( K_i \) value of indomethacin for inhibition of tolbutamide hydroxylase activity was 40 μM, although the inhibitory pattern was not demonstrated. This \( K_i \) value is similar to the \( K_M \) value (34.6 ± 5.4 μM) for indomethacin O-demethylation in human liver microsomes obtained in this study. If the inhibitory pattern for indomethacin inhibition of tolbutamide hydroxylase in the previous report is competitive, the coincidence of the \( K_M \) value with the \( K_i \) value would be reasonable.

Tolbutamide is a substrate of CYP2C9 (Veronese et al., 1991). (S)-Warfarin is primarily metabolized to 7-hydroxywarfarin by
CYP2C9 (Kaminsky and Zhang, 1997). Sulfaphenazole competitively inhibits tolbutamide hydroxylation (Miners et al., 1988) and (S)-warfarin 7-hydroxylation (Rettie et al., 1992) in human liver microsomes. Sulfaphenazole is a potent inhibitor of CYP2C9 (Baldwin et al., 1995). In the current study, these three compounds competitively inhibited indomethacin O-demethylation activity in human liver microsomes. The $K_M$ value for tolbutamide hydroxylase activity in human liver microsomes has been reported to be 62–176 μM (Veronese et al., 1991). The $K_M$ value for (S)-warfarin 7-hydroxylation activity in human liver microsomes has been reported to be 3.9 μM (Rettie et al., 1992). Thus, the $K_M$ values for tolbutamide and (S)-warfarin for inhibition of indomethacin O-demethylation obtained in this study were almost identical to the $K_M$ values for their own metabolic pathways.

In clinical use, the steady-state plasma concentration of indomethacin has been reported to be about 3 μM (Alván et al., 1975). Based on estimations of the indomethacin concentrations in liver, the indomethacin O-demethylation capability in microsomes of B lymphoblastoid cells expressing human CYPs was investigated with a 5 μM indomethacin concentration. Consequently, only cDNA-expressed CYP2C9 and CYP2C19 exhibited indomethacin O-demethylation activities. Because cDNA-expressed CYP2C9 exhibited greater activity than did cDNA-expressed CYP2C19, the kinetic parameters of these microsomes were investigated. The $K_M$ value for cDNA-expressed CYP2C9 was 12-fold lower than that for cDNA-expressed CYP2C19. The $V_{max}$ value for cDNA-expressed CYP2C9 was 1.4-fold higher than for cDNA-expressed CYP2C19. Therefore, the $V_{max}/K_M$ value for cDNA-expressed CYP2C9 was 16-fold higher than that for cDNA-expressed CYP2C19. In addition, it was reported that 60% of the CYP2C cDNA clones isolated from a human liver library were CYP2C9 and only 1% were CYP2C19 (Romkes et al., 1991). That report suggests that the expression level of the CYP2C9 isoform is higher than that of the CYP2C19 isoform. Considering those findings, the contribution of CYP2C19 to indomethacin O-demethylation activity in human liver microsomes was thought to be negligible. Thus, the results of this study suggest that CYP2C9 is primarily responsible for indomethacin O-demethylation in human liver microsomes.

We also determined the indomethacin O-demethylation activity in microsomes expressing the CYP2C9-Cys$^{144}$ variant. At 5 and 50 μM indomethacin concentrations, the activities were 0.048 and 0.097 pmol/min/pmol CYP, respectively. The isoform exhibited lower activity than did the major CYP2C9 type (i.e. CYP2C9-Arg$^{144}$) (fig. 3).

That finding agreed with previous findings that the CYP2C9-Cys$^{144}$ variant shows lower catalytic activity than does the major CYP2C9-Arg$^{144}$ type (Rettie et al., 1994).

Adverse interactions between indomethacin and warfarin have been reported (Chan and Critchley, 1994). In that report, it was proposed that the interaction mechanism involves displacement of warfarin from plasma protein binding sites. Indomethacin binds to serum proteins by as much as >90% (Mason and McQueen, 1974). Protein binding of warfarin has been reported to be 99% (Mungall et al., 1984). Displacement of a drug from protein binding sites results in modification of its pharmacokinetics. However, this is now not accepted as a potential mechanism (Rolan, 1994). When warfarin is displaced from protein the concentration of free drug is increased, but because more drug is available for elimination the concentration of free warfarin is soon lowered to its previous levels. Another NSAID, phenylbutazone, has been reported to affect (S)-warfarin metabolism (Lewis et al., 1974). It has been reported that the mechanisms of interaction of phenylbutazone and warfarin might involve metabolic interactions as well as displacement of protein binding (O’Reilly et al., 1980). In this study, it was clarified that indomethacin is metabolized by CYP2C9, which is responsible for 7-hydroxylation of (S)-warfarin. Therefore, it is possible that the interaction of indomethacin and warfarin might be due to metabolic interactions, rather than displacement of protein binding. Further study concerning the mechanism of the interaction between indomethacin and warfarin is underway in our laboratory.

CYP2C9 is known to be involved in the oxidative metabolism of many clinically used drugs, including tolbutamide (Veronese et al., 1991), phenytoin (Veronese et al., 1991), warfarin (Kaminsky and Zhang, 1997), and NSAIDs such as diclofenac (Leemann et al., 1993b), mefenamic acid (Bonnaby et al., 1994), and piroxicam (Kondo et al., 1992). In addition, CYP2C9 is inducible by rifampicin (LeClyse et al., 1996). Pharmacokinetic drug interaction studies by metabolic processes are regarded as the most important factors that affect the concentrations of drugs in the body. The findings obtained in this study suggest the possibility of the metabolic interaction of indomethacin with drugs that are metabolized by or are inducing for CYP2C9.

**References**


