Cyclooxygenase (COX) is an enzyme that catalyzes the first two steps in the biosynthesis of prostaglandins from arachidonic acid (Riendeau et al., 1997; Vane et al., 1998). Although it was long held that COX was a single enzyme present in most cells (Graul et al., 1997), more recent data have pointed to the existence of two forms (Vane et al., 1998). COX-I is the major form located in healthy tissues, and it plays a role in thrombogenesis and homeostasis of the gastrointestinal tract and kidneys (Smith and DeWitt, 1996). In contrast, COX-II is normally undetectable in most tissues and is inducible by cytokines, endotoxins, and mitogens. It has been associated with the elevated production of prostaglandins observed during inflammation, pain, and pyretic responses (Donnelly and Hawkey, 1997; Jouzeau et al., 1997; Lane, 1997; Lipsky and Isakson, 1997). Both forms of COX metabolize arachidonic acid via a similar mechanism, but they have different substrate specificities. COX-II accepts a wider range of substrates than COX-I (Battistini et al., 1994).

Most nonsteroidal anti-inflammatory drugs (NSAIDs) currently in use, such as indomethacin, ibuprofen, and diclofenac, inhibit both COX-I and COX-II with little or no selectivity for either form of the enzyme (Battistini et al., 1994; O’Neill et al., 1994). It is believed that NSAID-induced gastrointestinal damage and platelet and renal dysfunction result from the inhibition of COX-I, whereas the therapeutic benefit results from the inhibition of COX-II expressed at the sites of inflammation (Simon et al., 1998). Therefore, the identification and characterization of two isoforms of cyclooxygenase have stimulated tremendous efforts to develop potent and selective COX-II inhibitors (Penning et al., 1997; Simon et al., 1998). Celecoxib [Celebrex; SC-58635; 4-[5-(4-methylphenyl)-3-(trifluoromethyl)pyrazol-1-yl]benzenesulfonyl] is one example of a COX-II-selective agent (Fig. 1), and it has been approved by the Food and Drug Administration for the treatment of osteoarthritis and rheumatoid arthritis (Penning et al., 1997; Simon et al., 1998). Celecoxib is well absorbed and is extensively metabolized in humans, with less than 3% of the dose excreted unchanged.
CYP proteins.

Correlation analysis, and 4) cDNA-expressed human

CYP reaction phenotype of celecoxib remains to be
determined. Toward this end, the purpose of the present
study was to define the NADPH-dependent metabolism of
celecoxib in human liver microsomes (Mei et al., 1999). Other reagents
were purchased from commercial sources in the best available grade.

Incubation of Celecoxib with Native Human Liver Microsomes. In vitro incubations were carried out at 37°C in a Fisher
shaking water bath with 13 × 100-mm borosilicate glass disposable
culture tubes. The incubation mixture (final volume of 0.5 ml) con-
sisted of 0.1 M potassium phosphate buffer, pH 7.4, 10 mM MgCl₂, 1
mM EDTA, 1.0 mM NADP⁺, 10 mM D-glucose-6-phosphate, 2.0 U/ml
d-glucose-6-phosphate dehydrogenase (Sigma Type VII, from baker’s yeast), 0.10 to 0.20 mg/ml microsomal protein, and 0.5 to 50 μM
celecoxib dissolved in acetonitrile (0.3% v/v final concentration). The reaction was started by the addition of the NADPH-generating sys-

tem after a 5-min preincubation and was terminated with 2 ml of aceto-
nitrile. The internal standard, diclofenac (50 μl of a 100 μM stock), was added to the sample before centrifugation. The supernatant
was transferred to a clean tube and evaporated to dryness (SpeedVac; Savant Instruments, Inc., Holbrook, NY). In each case,
the residue was reconstituted in 150 μl of aqueous solution of ace-
tonitrile (30%) for HPLC analysis. Under these conditions, the rate of
hydroxy celecoxib formation was linear with respect to protein
concentration and time of incubation.

Measurement of CYP Form-Selective Activities. Microsomal
samples (Xenotech, LLC) were prepared for incubation and analysis
as described earlier. The rate of celecoxib (15 μM) hydroxylation was

correlated with the various CYP monoxygenase activities (data
provided by Xenotech, LLC), including 7-ethoxyresorufin O-deethyl-
ase (CYP1A2), coumarin 7-hydroxylase (CYP2A6), taxol 6-hydroxy-
lase (CYP2C8), tolbutamide methyl-hydroxylase (CYP2C9), (S)-me-

phenytoin 4'-hydroxylase (CYP2C19), chloroxazone 6-hydroxylase
(CYP2E1), bufuralol 1'-hydroxylase (CYP2D6), and testosterone 6β-
hydroxylase (CYP3A4/5). Correlation coefficients (r) were deter-
mined graphically and subjected to the Student’s t test (Rodrigues et
al., 1996).

CYP-Selective Inhibitors. Inhibition studies with CYP form-
selective chemical inhibitors were carried out at a final celecoxib
concentration of 5 μM (–Ki). Microsomal preparations from the
livers of three different subjects were used. SLF (0.1–10 μM,
CYP2C9), QND (0.1–10 μM, CYP2D6), and KTZ (0.1–10 μM,
CYP3A4) were dissolved in 50% (v/v) acetonitrile as stock solutions.
The inhibitors were individually incubated with each microsomal
sample (0.10 mg protein/ml) and substrate for 20 min. Control incu-
bations contained the same concentration of acetonitrile but no in-
hibitor (≤1% v/v final concentration). Inhibitor concentrations
were chosen on the basis of established Ki values (Bourrie et al., 1995;
Newton et al., 1995; Rodrigues et al., 1996) to ensure maximal
inhibition (>80%) of each CYP form (|ΔIC50| ≤ 10; |S| ≥ Ki).

Incubations with cDNA-Expressed Human CYP Micro-
somes. Incubations of celecoxib with various individual recombinant
CYP (rCYP) proteins were carried out as described for liver micro-
somes, except that the enzyme concentration was 5 or 25 nM.

For all CYP proteins tested, the reaction rates (picomoles per minute per picomole of CYP) were normalized (picomoles per minute
per picomole · picomoles of CYP per milligram) with respect to the

Materials and Methods

Chemicals and Biologicals. Ketoconazole (KTZ) and sulfa-
phenazole (SLF) were purchased from Research Biochemicals Inc.
(Natick, MA). Quinidine (QND) and diclofenac were obtained from
Sigma Chemical Co. (St. Louis, MO). The in-house preparations of
CYP2C9, CYP3A4, CYP1A2, CYP2C19, CYP2D6, and CYP2E1 were
used for kinetic studies or the determination of reaction rates. The
procedures for their preparation have been described elsewhere (Mei
et al., 1999). Individual and pooled human liver microsomal samples
were purchased from the Gentest Corporation (Woburn, MA) and
The International Institute for the Advancement of Medicine (Exton,
PA). A bank of fully characterized human liver microsomes (n = 16
different organ donors) was purchased from Xenotech LLC (Kansas
City, KS). Mouse ascites containing monoclonal antibodies (mAbs)
raised against CYP2C9 (mAb2C9a) and CYP3A4 (mAb3A4a) were
prepared in-house. The antibodies have been characterized with
respect to their CYP selectivity (Mei et al., 1999). Other reagents
were purchased from commercial sources in the best available grade.

Fig. 1. Proposed major celecoxib oxidative pathway.
in urine and feces (Karim et al., 1997). The major route of
metabolism appears to be methylation hydroxylation, with further
oxidation to the corresponding carboxylic acid (Fig. 1). Al-
though CYP2C9 has been implicated (Karim et al., 1997), a
complete CYP reaction phenotype of celecoxib remains to be
determined. Toward this end, the purpose of the present
study was to define the NADPH-dependent metabolism of
celecoxib in human liver microsomes and to determine which
CYP protein or proteins were involved, using a combination of
four approaches: 1) immunoinhibition, 2) chemical inhibi-
tion, 3) correlation analysis, and 4) cDNA-expressed human
CYP proteins.
Results

Metabolism of Celecoxib. A typical UV chromatogram of the extract after incubation of celecoxib with NADPH-fortified human liver microsomes is shown in Fig. 2. After incubation, one major metabolite (retention time, 10.2 min) was detected and identified (LC-MS/MS) as hydroxy celecoxib (Fig. 1). No carboxy celecoxib was detected, although minor amounts of the corresponding aldehyde were detected by LC-MS (data not shown).

Overall, the formation of methyl hydroxy celecoxib conformed to saturable kinetics, and a representative Michaelis-Menten plot (pool of n = 10 different livers) is presented in Fig. 3. A slight but significant decrease in hydroxy celecoxib formation was observed at higher celecoxib concentrations. Similar results were obtained with microsomal preparations of three individual livers (data not shown). Therefore, only the data acquired at substrate concentrations of ≤20 μM were fitted to the simple Michaelis-Menten equation for the determination of kinetic parameters (Table 1). Eadie-Hofstee plots of those data indicated that hydroxylation of celecoxib in human liver microsomes exhibited monophasic enzyme kinetics over the substrate concentration range of 1 to 20 μM (Fig. 3, inset).

Immunoinhibition of Hydroxy Celecoxib Formation.

The mAb against human CYP2C9 significantly inhibited the formation of hydroxy celecoxib in human liver microsomes, yielding up to >85% inhibition in a concentration-dependent manner (Fig. 4A). The mAbs against human CYP3A4 suppressed hydroxy celecoxib formation by ~10% at low concentrations, and its inhibitory effect diminished as the concentration of ascites increased. When the volume of ascites...
reached >5 μL, a small but significant rise in the rate of hydroxy celecoxib formation was observed (Fig. 4B). To avoid stimulation of celecoxib hydroxylase activity, therefore, the ascites fluid containing anti-CYP3A4 was diluted (1:32) in subsequent experiments. The presence of both antibodies in the incubation system almost completely inhibited the formation of hydroxy celecoxib (90–95% inhibition) as shown in Fig. 4C.

Correlation Studies. The formation rates of hydroxy celecoxib were determined in 16 different human liver microsomal preparations. There was considerable interindividual variability in the values obtained with a mean ± S.D. activity (nanomoles per minute per milligram of protein) of 0.48 ± 0.17. As shown in Fig. 6, A and B, the rate of hydroxy celecoxib formation correlated with tolbutamide methyl-hydroxylation (CYP2C9) and testosterone 6β-hydroxylation (CYP3A4/5) activities (r = 0.92, P < .01 and r = 0.55, P < .05, respectively). Interestingly, the suppression of one of these CYPs by its corresponding antibody improved the correlation with the second CYP form. Namely, inhibition of celecoxib hydroxylation by antibody against CYP3A4 slightly improved the correlation with tolbutamide methyl-hydroxylase activity (r = 0.93, P < .005, Fig. 6C). However, inhibition of celecoxib hydroxylation by antibody against CYP2C9 significantly enhanced the correlation with testosterone 6β-hydroxylase activity (r = 0.96, P < .005, Fig. 6D). The correlation of hydroxy celecoxib formation with activities selective for other CYP isoforms [e.g., 7-ethoxyresorufin O-dealkylation, CYP1A2; coumarin 7-hydroxylation, CYP2A6; 7-ethoxy-4-trifluoro-methylcoumarin O-deethylation, CYP2B6; taxol-6-hydroxylation, CYP2C8; (S)-mephenytoin 4′-hydroxylation, CYP2C19; dextromethorphan-O-demethylation, CYP2D6; and chlorzoxazone 6-hydroxylation, CYP2E1] was not statistically significant (Table 2).

Chemical Inhibition. Based on the immunoinhibition and correlation data, three selective chemical inhibitors (SLF, KTZ, and QND) were chosen to substantiate the contribution of CYP2C9 and CYP3A4 in the metabolism of celecoxib. As displayed in Fig. 7, SLF (selective for CYP2C9) and KTZ (selective for CYP3A4) significantly inhibited the formation of hydroxy celecoxib in a concentration-dependent manner, whereas QND (selective for CYP2D6) did not show any significant effect (inhibition, <5%).

Celecoxib Metabolism by cDNA-Expressed Human Cytochromes P450. The CYP reaction phenotype of celecoxib was further evaluated with rCYP proteins. Consistent

### Table 1

<table>
<thead>
<tr>
<th>Microsome Preparation</th>
<th>K&lt;sub&gt;m&lt;/sub&gt;</th>
<th>V&lt;sub&gt;max&lt;/sub&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>HHM0259&lt;sup&gt;a&lt;/sup&gt;</td>
<td>5.1</td>
<td>0.43</td>
</tr>
<tr>
<td>HG30</td>
<td>2.8</td>
<td>1.37</td>
</tr>
<tr>
<td>HG56</td>
<td>3.4</td>
<td>0.55</td>
</tr>
<tr>
<td>HG66</td>
<td>3.9</td>
<td>0.45</td>
</tr>
<tr>
<td>Mean (S.D.)</td>
<td>3.8 (0.9)</td>
<td>0.70 (0.45)</td>
</tr>
</tbody>
</table>

<sup>a</sup> Pool of livers (n = 10 different subjects).
with the results obtained with native human liver microsomes, a decrease in celecoxib methyl hydroxylase activity was also observed when the substrate concentrations exceeded 15 μM (Fig. 8A). Fitting the data acquired at substrate concentrations of ≤15 μM yielded an apparent $K_m$ value of 4.1 μM, which was comparable to that observed with native human liver microsomes. By comparison, the rCYP3A4-catalyzed metabolism of celecoxib was best described by a simple Michaelis-Menten equation and was characterized by a higher $K_m$ value (18 versus 4.1 μM) and a lower $V_{max}$ value (1.4 versus 17 pmol/min/pmol rCYP) compared with rCYP2C9 (Fig. 8B).

The rates of hydroxy celecoxib formation in the presence of several rCYPs are listed in Table 3. On normalization of the data with respect to the abundance of each enzyme in native human liver microsomes (Rodrigues, 1999), it was found that the majority (86%) of the hydroxylase activity was attributable to CYP2C9, although CYP3A (6%) did contribute. The normalized data were in agreement with the results of the immunoinhibition experiments.

**Discussion**

In the present study, it has been shown that the first step in the hepatic biotransformation of celecoxib (≤25 μM) involves a methyl hydroxylation reaction mediated principally by CYP2C9. The involvement of CYP2C9 is well supported by several lines of evidence: 1) a good correlation between the rate of hydroxy celecoxib formation and tolbutamide hydroxylase activity in a panel of human liver microsomes, 2) extensive inhibition of celecoxib metabolism by mAbs against CYP2C9 and SLF (a selective inhibitor of CYP2C9), and 3) a high turnover in the presence of human rCYP2C9. However, based on the results of this study, it is concluded that a member or members of the human liver microsomal CYP3A subfamily play a minor role (<10%). For example, the correlation of celecoxib hydroxylase with testosterone 6β-hydroxylase was improved in the presence of anti-CYP2C9 (r = 0.96 versus 0.55). In addition, KTZ at 0.1 μM (a specific inhibitor of CYP3A) and anti-CYP3A4 mAbs significantly inhibited the formation of hydroxy celecoxib by 10%. The greater inhibitory effect seen at a higher concentration of KTZ (≥1 μM) may be attributable to the nonselective inhibition of CYP2C9 (Boobis, 1995).

The involvement of other CYP forms in the metabolism of celecoxib is unlikely. rCYP2D6 showed considerable activity (2.9 pmol/min/pmol rCYP2D6), but there was no correlation between celecoxib metabolism and dextromethorphan O-demethylase activity and no inhibition in the presence of QND.
(a selective inhibitor of CYP2D6) in native human liver microsomes. Similarly, although appreciable activity was observed with rCYP2C19 (3.2 pmol/min/pmol rCYP2C19), the poor correlation between celecoxib hydroxylase and (S)-mephentyoin 4'-hydroxylase in human liver microsomes did not support the involvement of the enzyme. However, it is possible that CYP2C19 may play a more prominent role in some individuals with higher CYP2C19 levels (Lasker et al., 1998).

Moreover, metabolism by CYP2C8 in human liver microsomes was ruled out because of the weak correlation with taxol hydroxylase activity (Table 2). It should be noted that SLF would not be expected to inhibit (≤10%) CYP2C8 and CYP2C19 at the concentrations used in the present study (Mancy et al., 1996).

The decrease in hydroxy celecoxib formation rate at higher substrate concentrations may be suggestive of substrate inhibition and the fact that simple Michaelis-Menten kinetics do not adequately model the kinetic data. In fact, the experimental data were best modeled by incorporation of a term for substrate inhibition. Unfortunately, the poor aqueous solubility of celecoxib prevented the use of high substrate concentrations, and it was not possible to better define the inhibition term. By comparison, other investigators have been able to use a wider range of substrate concentrations to

### Table 2: Correlation of various CYP activities with the formation of hydroxy celecoxib in human liver microsomes

<table>
<thead>
<tr>
<th>Activity</th>
<th>CYP</th>
<th>Correlation Coefficient ($r^2$)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>7-Ethoxyresoruifen O-dealkylation</td>
<td>1A2</td>
<td>0.26</td>
<td>&gt;.3</td>
</tr>
<tr>
<td>Coumarin 7-hydroxylation</td>
<td>2A6</td>
<td>0.46</td>
<td>&gt;.05</td>
</tr>
<tr>
<td>7-EFC O-dealkylation</td>
<td>2B6</td>
<td>0.29</td>
<td>&gt;.2</td>
</tr>
<tr>
<td>(S)-Mephytoin 4'-hydroxylation</td>
<td>2C19</td>
<td>0.35</td>
<td>&gt;.2</td>
</tr>
<tr>
<td>Taxol-6-hydroxylation</td>
<td>2C8</td>
<td>0.42</td>
<td>&gt;.1</td>
</tr>
<tr>
<td>Tolbutamide methyl-hydroxylation</td>
<td>2C9</td>
<td>0.92 (0.93)$^a$</td>
<td>&lt;.001 (&lt;.001)</td>
</tr>
<tr>
<td>Dextromethorphan O-demethylation</td>
<td>2D6</td>
<td>0.11</td>
<td>&gt;.6</td>
</tr>
<tr>
<td>Chlorzoxazone 6-hydroxylation</td>
<td>2E1</td>
<td>0.35</td>
<td>&gt;.1</td>
</tr>
<tr>
<td>Testosterone 6β-hydroxylation</td>
<td>3A4/5</td>
<td>0.55 (0.96)$^a$</td>
<td>&lt;.02 (&gt;.001)</td>
</tr>
</tbody>
</table>

$^a$ Panel consisted of microsomal preparations from 16 different human livers.
$^b$ 7-EFC, 7-ethoxy-4-trifluoromethylcoumarin.
$^c$ Hydroxy celecoxib formation was determined in the presence of anti-CYP3A4 ascites.
$^d$ Hydroxy celecoxib formation was determined in the presence of anti-CYP2C9 ascites.

### Table 3: Formation of hydroxy celecoxib by recombinant CYP proteins expressed in insect cells

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Hydroxy Celecoxib Formation Rate</th>
<th>Mean Specific CYP Content</th>
<th>Normalized Hydroxy Celecoxib Formation Rate</th>
<th>Percentage of Total Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>CYP2C9</td>
<td>12.30</td>
<td>0.096</td>
<td>1.18</td>
<td>86.1</td>
</tr>
<tr>
<td>CYP3A4</td>
<td>0.70</td>
<td>0.108</td>
<td>0.08</td>
<td>5.8</td>
</tr>
<tr>
<td>CYP1A2</td>
<td>0.42</td>
<td>0.045</td>
<td>0.02</td>
<td>1.4</td>
</tr>
<tr>
<td>CYP2C19</td>
<td>3.19</td>
<td>0.019</td>
<td>0.06</td>
<td>4.4</td>
</tr>
<tr>
<td>CYP2D6</td>
<td>2.90</td>
<td>0.010</td>
<td>0.03</td>
<td>2.1</td>
</tr>
<tr>
<td>CYP2E1</td>
<td>0.02</td>
<td>0.049</td>
<td>0.00</td>
<td>0</td>
</tr>
<tr>
<td>Total</td>
<td>1.37</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

$^a$ 5 pmol rCYP/ml and 25 pmol rCYP/ml for CYP2C9 and other isoforms, respectively. Incubation at 37°C for 30 min (celecoxib concentration of 25 μM).
$^b$ Mean specific content of CYP in native human liver microsomes (Rodrigues, 1999).
$^c$ Rate of hydroxy celecoxib formation normalized with respect to the specific content of each CYP in native human liver microsomes.

![Fig. 7](image-url) Effects of different concentrations of chemical inhibitors on human liver microsome-catalyzed celecoxib methyl hydroxylase activity (celecoxib was evaluated at a final concentration of 5 μM). Reaction rates are expressed as a percentage of the control velocity without inhibitor (mean ± S.D., n = 3 microsomal preparations).

![Fig. 8](image-url) Rates of formation of hydroxy celecoxib by the recombinant CYP2C9 (A) and CYP3A4 (B). Insets, Eadie-Hofstee plots.
succefully evaluate substrate inhibition (Goeger and Anderson, 1992; Gorski et al., 1994; Tucker et al., 1994; Schmider et al., 1996; von Moltke et al., 1996; Shiraga et al., 1999). Interestingly, substrate inhibition was limited to celecoxib hydroxylase activity catalyzed by CYP2C9 and was not observed with CYP3A4. Moreover, the phenomenon was observed with the recombinant enzyme and native liver microsomes. To our knowledge, celecoxib is the first CYP2C9 substrate that exhibits substrate inhibition; no similar findings have been reported for commonly used CYP2C9 probes, such as tolbutamide, diclofenac, and phenytoin.

Although CYP2C9 and CYP3A4 play a role in the metabolism of celecoxib, it is anticipated that celecoxib will not significantly interfere with the metabolism of other drugs catalyzed by CYP3A4. On the other hand, interaction with drugs metabolized by CYP2C9 is possible because of the relatively high affinity of celecoxib for the enzyme. Because CYP2C9 will largely govern celecoxib clearance at clinically relevant concentrations, the coadministration of other CYP2C9 inhibitors or inducers is likely to alter celecoxib clearance. For instance, fluconazole has been shown to increase the area under the curve of celecoxib (≈2-fold) (Celebrex package insert). Because fluconazole is a known CYP2C9 inhibitor (Kunze et al., 1996), these data attest to the role of the enzyme in the overall clearance of celecoxib. In addition, the pharmacokinetics of celecoxib will be dependent on CYP2C9 genotype, especially in subjects genotyped homozygous for the allelic variant forms (e.g., CYP2C9*1/*1 or CYP2C9*3/*3) of the enzyme (C. Tang, M. Shou, T. H. Rushmore, and A. D. Rodrigues, unpublished data; Miners and Birckett, 1998). It is important to note that CYP2C9 is one of the most abundant CYP enzymes in the human liver and has been shown to metabolize a large number of drugs, including tolbutamide, phenytoin, (S)-warfarin, losartan, sulfamethoxazole, and various NSAIDs (Goldstein and de Moraes, 1994; Lasker et al., 1998; Miners and Birckett, 1998; Gill et al., 1999; McCrea et al., 1999). In summary, the results of the present study indicate that celecoxib methyl hydroxylation is largely catalyzed by CYP2C9, although CYP3A4 plays a minor role.

References

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