Confirmation That Cytochrome P450 2C8 (CYP2C8) Plays a Minor Role in (S)-(++)- and (R)-(−)-Ibuprofen Hydroxylation in Vitro

Shu-Ying Chang, Wenyong Li, Sarah C. Traeger, Bei Wang, Donghui Cui, Hongjian Zhang, Bo Wen, and A. David Rodrigues

Bristol-Myers Squibb, Princeton, New Jersey (S.-Y.C., W.L., S.C.T., B.W., A.D.R.); Merck Research Laboratories, West Point, Pennsylvania (D.C.); Roche, Palo Alto, California (B.W.); and PharmaResources Co., Shanghai, China (H.Z.)

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ABSTRACT:

Various groups have sought to determine the impact of CYP2C8 genotype (and CYP2C8 inhibition) on the pharmacokinetics (PK) of ibuprofen (IBU) enantiomers. However, the contribution of cytochrome P450 2C8 (CYP2C8) in human liver microsomes (HLMs) has not been reported. Therefore, in vitro cytochrome P450 (P450) reaction phenotyping was conducted with selective inhibitors of cytochrome P450 2C9 (CYP2C9) and CYP2C8. In the presence of HLMs, sulfaphenazole (CYP2C9 inhibitor), and anti-CYP2C9 monoclonal antibodies (mAbs) inhibited (73–100%) the 2- and 3-hydroxylation of both IBU enantiomers (1 and 20 μM), the same inhibitors were less able to inhibit the 2-hydroxylation of (S)-(++)-IBU (32–52%) and (R)-(−)-IBU (30–64%), whereas the 3-hydroxylation of (S)-(++)-IBU and (R)-(−)-IBU was inhibited 68 to 83 and 70 to 89%, respectively. In contrast, less inhibition was observed with montelukast (CYP2C8 inhibitor, ≤35%) and anti-CYP2C8 mAbs (≤24%) at all concentrations of IBU. When (S)-(++)-IBU and (R)-(−)-IBU (1 μM) were incubated with a panel of recombinant human P450s, only CYP2C9 formed appreciable amounts of the hydroxy metabolites. At a higher IBU enantiomer concentration (500 μM), additional P450s catalyzed 2-hydroxylation (CYP3A4, CYP2C8, CYP2C19, CYP2D6, CYP2E1, and CYP2B6) and 3-hydroxylation (CYP2C19). When the P450 reaction phenotype and additional clearance pathways are considered (e.g., direct glucuronidation and chiral inversion), it is concluded that CYP2C8 plays a minor role in (R)-(−)-IBU (<10%) and (S)-(++)-IBU (<13%) clearance. By extension, one would not expect CYP2C8 inhibition (and genotype) to greatly affect the pharmacokinetic profile of either enantiomer. On the other hand, CYP2C9 inhibition and genotype are expected to have an impact on the PK of (S)-(++)-IBU.

There has been continued interest in the safety and ADME properties of cyclooxygenase inhibitors. This interest has extended to 2-(4-isobutylphenyl)propionic acid (ibuprofen, IBU), which is administered as a racemic mixture of (S)-(++)-IBU and (R)-(−)-IBU. There are two metabolites of IBU, the 2-hydroxyibuprofen (32–52%) and 3-hydroxyibuprofen (30–64%), whereas the 3-hydroxylation of (S)-(++)-IBU and (R)-(−)-IBU was inhibited 68 to 83 and 70 to 89%, respectively. In contrast, less inhibition was observed with montelukast (CYP2C8 inhibitor, ≤35%) and anti-CYP2C8 mAbs (≤24%) at all concentrations of IBU. When (S)-(++)-IBU and (R)-(−)-IBU (1 μM) were incubated with a panel of recombinant human P450s, only CYP2C9 formed appreciable amounts of the hydroxy metabolites. At a higher IBU enantiomer concentration (500 μM), additional P450s catalyzed 2-hydroxylation (CYP3A4, CYP2C8, CYP2C19, CYP2D6, CYP2E1, and CYP2B6) and 3-hydroxylation (CYP2C19). When the P450 reaction phenotype and additional clearance pathways are considered (e.g., direct glucuronidation and chiral inversion), it is concluded that CYP2C8 plays a minor role in (R)-(−)-IBU (<10%) and (S)-(++)-IBU (<13%) clearance. By extension, one would not expect CYP2C8 inhibition (and genotype) to greatly affect the pharmacokinetic profile of either enantiomer. On the other hand, CYP2C9 inhibition and genotype are expected to have an impact on the PK of (S)-(++)-IBU.

ABBREVIATIONS: ADME, absorption-distribution-metabolism-excretion; IBU, 2-(4-isobutylphenyl)propionic acid, ibuprofen; P450, cytochrome P450; rCYP2C8, recombinant cytochrome P450 2C8; rCYP2C9, recombinant cytochrome P450 2C9; PK, pharmacokinetics; mAb, monoclonal antibody; HLM, human liver microsomes; 2-OH, 2-hydroxyibuprofen; 3-carboxy-IBU, 3-(4-(1-carboxyethyl)phenyl)-2-methylpropanoic acid, 3-carboxyibuprofen; 3-OH, 2-(4-(3-hydroxy-2-methylpropyl)phenyl)-propanoic acid, 3-hydroxyibuprofen; HPLC, high-performance liquid chromatography; NR, normalized rate; TNR, total normalized rate; LC-MS/MS, liquid chromatography-tandem mass spectrometry; CLint, intrinsic clearance (Vmax/Km); OATP, organic anion-transporting peptide; AUC, area under the plasma concentration versus time curve.
itors and inhibitory mAbs were not available when Hamman et al. (1997) conducted their in vitro P450 reaction phenotyping study. Therefore, the CYP2C8-catalyzed 2- and 3-hydroxylation of individual IBU enantiomers in HLMs warranted further study, and the role of CYP2C9 and CYP2C8 therein was assessed using available selective inhibitors of each P450 form (chemicals and antibodies). Both enantiomers were also incubated with a panel of recombinant human P450s, and the data were integrated with the inhibition profile obtained with HLMs. Throughout the present study, a wide range of \((S)-(\text{H11001})\)-IBU and \((R)-(\text{H11002})\)-IBU concentrations was used (1–500 \(\mu\text{M}\)). Such a range encompasses the clinically relevant free (\(1\mu\text{M}\)) and total (\(100\mu\text{M}\)) plasma concentrations of each enantiomer (Davies, 1998; Andersson et al., 2004; Takanohashi et al., 2007). Integration of the above in vitro data, with known human ADME information, may help rationalize reports describing IBU PK-P450 inhibitor combinations and IBU PK-P450 genotype associations (Kirchheiner et al., 2002; Garcia-Martin et al., 2004; Martinez et al., 2005; Hynninen et al., 2006; Bell et al., 2007; Tornio et al., 2007).

Materials and Methods

Materials. \((S)+(\text{H11001})\)- and \((R)-(\text{H11002})\)-enantiomers of IBU were purchased from BIOMOL Research Laboratories (Plymouth Meeting, PA). 2-OH (racemic) authentic standard and 3-(4-(1-carboxyethyl)phenyl)-2-methylpropanoic acid (3-carboxy-IBU) \([\text{racemate for synthesis of } 2-(4-(3\text{-hydroxy}-2\text{-methylpropyl})\text{-phenyl})\text{-propanoic acid (3-OH)}]\) were obtained from Toronto Research Chemicals (North York, ON, Canada). Sulfaphenazole and 7-hydroxycoumarin (internal standard) were purchased from Sigma-Aldrich (St. Louis, MO). Montelukast was also obtained from a commercial source (Sequoia Research Products, Pangbourne, UK). Ascites fluid containing mAbs selective for CYP2C8 (preparation 281-1-1), CYP2C9 (preparation 763-15-5), and hen egg white lysozyme (preparation HyHel-9) were purchased by special order from the Laboratory of Molecular Carcinogenesis and Laboratory of Metabolism (National Cancer Institute, National Institutes of Health, Bethesda, MD). Pooled HLMs \((n = 27\text{ different organ donors})\) and baculovirus-infected insect cell microsomes (Supersomes) containing various cDNA-expressed human P450s \((\text{coexpressed with P450 oxidoreductase})\) were purchased from BD Gentest (Woburn, MA). HLM preparations from 12 individual \((\text{CYP2C9 and CYP2C8 genotyped})\) organ donors were purchased by special order from BD Gentest. All HLM preparations were characterized in terms of total P450, cytochrome \(c\) reductase activity, diclofenac 4'-hydroxylase \((\text{CYP2C9})\), paclitaxel 6a-hydroxylase \((\text{CYP2C8})\), and testosterone 6\(\beta\)-hydroxylase \((\text{CYP3A})\) activity. All other reagents and chemicals were of analytical grade and the highest quality available commercially.

3-OH Synthesis, Isolation, and Structure Confirmation. Synthesis. 3-OH was synthesized using a three-step procedure. First, 3-carboxy-IBU (35 mg, 0.148 mmol) was dissolved in ethanol (5 ml). The solution was refluxed for 2.5 h after the addition of 1 drop of sulfuric acid. After the reaction was completed, the reaction solvent was removed under reduced pressure, and the residue was diluted with dichloromethane and saturated sodium bicarbonate aqueous solution. The product was extracted with dichloromethane twice \((\text{two }15\text{-ml washes})\), dried over sodium sulfate, and concentrated to dryness to give the diester as a white solid \((40\text{ mg, }92\% \text{ yield; } mlc = 315.35 \text{ (M + 23)})\).
TABLE 1

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<th>Kinetic data describing the 2- and 3-hydroxylation of IBU enantiomers in the presence of rCYP2C8, rCYP2C9, and pooled HLMs</th>
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<td>Estimates of $K_v$, $K_m$, and $V_{max}$ were determined graphically (see biphasic model. S.E. and $CL_{int}$) is the ratio $K_v$ to $V_{max}$ and describes the low $K_v$ component. It was not possible to determine the $K_v$ and $V_{max}$ components.</td>
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<td>Second, the diester (40 mg, 0.137 mmol) was dissolved in tetrahydrofuran and 1 M LiAlH$_4$ (in tetrahydrofuran) was added dropwise at room temperature. After the addition of 45 drops of LiAlH$_4$ (0.6 ml, 0.6 mmol), HPLC (at 220 nm) showed that the ratio of diol [3-(4-(1-hydroxypropan-2-yl)phenyl)-2-methylpropanoic acid] and monoesters [ethyl 2-(4-(3-hydroxy-2-methylpropyl)phenyl)-propanoate and ethyl 3-(4-(1-hydroxypropan-2-yl)phenyl)-2-methylpropanoate], and starting diester was 5:3:2:5. The reaction was quenched with 1 N HCl and extracted with dichloromethane. The organic layer was concentrated under reduced pressure, and then the residue was purified by flash chromatography to give a mixture of the monoesters as white solids, which could not be separated by column chromatography [$m/z = 273.35$ (M + $23$)].</td>
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<td>Third, the mixture of the monoesters was dissolved in methanol (2 ml), and 2 drops of 50 wt% sodium hydroxide aqueous solution and water (1 ml) was added. The solution was stirred at room temperature for 2 h and then acidified with 1 N HCl to pH &lt;4, and the resulting products were extracted with dichloromethane (two 10-ml washes). The organic layer was dried over sodium sulfate and concentrated to dryness to give 3-OH and 3-(4-(1-hydroxypropan-2-yl)phenyl)-2-methylpropanoic acid [8 mg, 0.035 mmol; $m/z = 221.20$ (M – 1)].</td>
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<td>Isolation. The residue containing 3-OH and 3-(4-(1-hydroxypropan-2-yl)-phenyl)-2-methylpropanoic acid (isomer) was dissolved in 0.8 ml of methanol. The methanol solution was subjected to semipreparative HPLC: column, YMC ProC$_{18}$, 20 × 150 mm, S$_5$ (Waters, Milford, MA); mobile phase, 0.1% formic acid in water(A)/0.1% formic acid in acetonitrile (B); and gradient, 10 to 27% B in 5 min and 27% B for 30 min. The flow rate was 10 ml/min with UV detection at 220 nm. Under these conditions, 3-OH and the isomer eluted at approximately 24 and 22 min, respectively. Fractions containing 3-OH and the isomer were pooled separately and lyophilized. After lyophilization, 3-OH (0.5 mg) and isomer (3 mg) were obtained as a white powder and the former was submitted for NMR analysis.</td>
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<td>Structure confirmation. NMR data were collected on a JEOL Eclipse spectrometer operating at 500.16 MHz resonance frequency and fitted with a 5-mm auto tune Z-gradient broadband probe. Samples (0.5 mg) of the reputed 3-OH and undesired synthetic isomer (3.0 mg) were each dissolved in 180 μl of 99.95% d$_4$-methanol (Cambridge Isotope Laboratories, Inc., Andover, MA) and placed in a 3-mm NMR tube. One- and two-dimensional 1H and 13C NMR experiments ($^1$H, $^{13}$C, distortionless enhancement of polarization transfer using a 135 degree decoupler pulse, correlation spectroscopy, $^{1}$H-$^{13}$C heteronuclear multiple quantum coherence spectroscopy, and $^{1}$H-$^{13}$C heteronuclear multiple-bond correlation spectroscopy) were performed on both samples. The complete $^1$H and $^{13}$C chemical shift assignment was accomplished by detailed analysis of the correlated spectra. The relative and absolute stereochemistry of the two chiral centers was not confirmed.</td>
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<td>For 3-OH, the complete $^{1}$H-$^{1}$H spin coupling systems of the 3-hydroxy-2-methylpropyl moiety and propanoic acid moiety were observed in the correlation spectroscopy spectrum. $^{1}$H-$^{13}$C long-range correlations from both the doublet methyl (0.86 ppm) and the benzylidene methane protons (2.75 and 2.32 ppm) of the terminal hydroxyl methylene group at 68.0 ppm were observed. $^{1}$H NMR (500 MHz, methanol-d$_4$) δ 8.46 (1H, br s), 7.22 (2H, d, $J$ = 7.7 Hz), 7.12 (2H, d, $J$ = 7.7 Hz), 3.66 (1H, q, $J$ = 7.2 Hz), 3.42 (1H, dd, $J$ = 11.0, 6.0 Hz), 3.36 (1H, dd, $J$ = 11.0, 6.6 Hz), 2.75 (1H, dd, $J$ = 13.5, 5.8 Hz), 2.32 (1H, dd, $J$ = 13.5, 8.5 Hz), 1.86 (1H, dd, $J$ = 14.7, 6.5, 6.3, 6.0 Hz), 1.43 (3H, d, $J$ = 7.2 Hz), 0.86 (3H, d, $J$ = 6.6 Hz). $^{13}$C NMR (126 MHz, methanol-d$_4$) δ 179.4, 140.9, 140.5, 130.5 (2C), 128.5 (2C), 68.0, 47.0, 40.4, 39.3, 19.4, 16.9.</td>
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<td>Incubation of IBU Enantiomers with Recombinant P450 Proteins and HLMs. Incubations were carried out in 96-well Thermowell Gold plates (Corning Inc., Corning, NY) using an 8-tip Genesis 150 liquid handler (Tecan Group Ltd., Research Triangle Park, NC) equipped with a temperature-controlled shaker. The incubation mixture (final volume of 0.2 ml) at 37°C consisted of the following: 0.1 M potassium phosphate buffer (pH 7.4), MgCl$_2$ (2.5 mM), protein (HLMs, 0.05 mg/ml; recombinant P450, 1.25 mmol/ml), and individual IBU enantiomer (1–1000 μM) dissolved in acetonitrile (1.0%, v/v, final concentration). After a 5-min prewarming, the reaction was started with NADPH (2.4 mM). Routinely, the reaction was terminated at 20 to 30 min by transferring 120 μl of incubation mixture into a MultiScreen filter plate (Millipore Corporation, Billerica, MA) preloaded with 240 μl of ice-cold acetonitrile containing 7-hydroxyxocoumarin (1 μM final concentration). All reactions were linear with respect to time and protein (or enzyme) concentration.</td>
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For each recombinant P450 form, the normalized rate (NR) was calculated based on the turnover (picomoles per minute per picomole of P450) and the reported specific content of each P450 form in HLMs (picomoles per milligram). The total normalized rate (TNR) was calculated as the sum of the NR values for each P450. For each P450 form, the NR was expressed as a percentage of the TNR (Rodrigues, 1999). Representative (mean) specific contents were used for CYP3A4, CYP2E1, CYP2D6, CYP2C19, CYP1A2, CYP2C9, CYP2C8, CYP2B6, and CYP2A6 (108, 49, 10, 19, 45, 96, 64, 39, and 41 pmol/mg, respectively) (Lasker et al., 1998; Rodrigues, 1999; Lapple et al., 2003; Zhang et al., 2007).

Identification and Quantitation of 2-OH and 3-OH. After the addition of cold acetonitrile to the incubates, the filter plate (above) was stacked with a 2-ml 96-well plate (VWR, West Chester, PA) preloaded with water (360 µl) and then vortexed and centrifuged at 2000 g (5 min). Approximately 10 µl of supernatant was analyzed by LC-MS/MS using an API4000 LC-MS/MS tandem mass spectrometer (Applied Biosystems, Streetsville, ON, Canada) equipped with a turbo-V source, two LC-10ADvp pumps with an SCL-10ADvp controller and DGU-14 solvent degasser (Shimadzu, Columbia, MD), and a CTC PAL autosampler (LEAP Technologies, Carrboro, NC). A Luna phenyl heptyl column (150 x 2.0 mm; Phenomenex, Torrance, CA) was used to achieve HPLC separation. The column was maintained at room temperature with a gradient elution at a flow rate of 0.3 ml/min and mobile phase A (0.1%
formic acid in water) and B (0.1% formic acid in acetonitrile). 2-OH and 3-OH were chromatographically separated using a gradient of 10 to 60% B (over 5 min) followed by a gradient to 100% B (over 2.5 min). Retention times were approximately 4.3, 4.7, and 4.8 min for 7-hydroxycoumarin, 2-OH, and 3-OH, respectively. The mass spectrometer was operated under atmospheric pressure ionization, in the negative ion (multiple reaction monitoring) mode. Ion transitions were monitored for 2-OH and 3-OH (m/z 161→177) and 7-hydroxycoumarin (m/z 161→133). The product ion scan (m/z 110→240) for 2-OH and 3-OH (parent m/z 221) was obtained to confirm the structure of the HLM metabolites found at retention times 4.7 and 4.8 min, respectively. The spectra associated with the metabolite peaks detected in HLM incubates matched the synthesized 3-OH and the commercially available 2-OH authentic standard.

For the two IBU hydroxy metabolites, mass detections were acquired and quantitative analysis was performed using Analyst software version 1.4.2 (Applied Biosystems). In each case, the analyte/7-hydroxycoumarin peak area ratios of the calibrators were fitted to a quadratic regression model (weighted 1/x). Calibration standards were prepared in HLMs and extracted with acetonitrile in the same manner as the samples (above). A 6-point standard curve (0.1–2000 nM in duplicate) was set up for 2-OH and was fitted to a quadratic regression model (weighted 1/x correlation coefficient ≥0.99). The concentration of 2-OH and 3-OH in each sample was calculated using this 2-OH standard curve. No attempt was made to separate and quantitate the two individual diastereomeric forms of 3-OH [e.g., (R,S)-3-OH and (R,R)-3-OH].

Kinetic Studies Involving HLMs, rCYP2C8, and rCYP2C9. Kinetic parameters describing the 2- and 3-hydroxylation of each IBU enantiomer were determined after incubation with HLMs and recombinant P450s as described above. However, a range of individual IBU enantiomer (nominal) concentrations was used (1, 2, 4, 8, 16, 32, 63, 125, 250, and 500 μM). Rates of 2-hydroxylation and 3-hydroxylation were determined and the untransformed data were analyzed using nonlinear methods with KaleidaGraph 3.6 (Synergy Software, Reading, PA). The data were fit to numerous models (Korzekwa et al., 1998; Shou et al., 2005a,b; Zhang et al., 2007).

Inhibition Studies. Inhibition studies with P450 form-selective chemical inhibitors were carried out as described above, at a final IBU enantiomer concentration of 1, 20, and 500 μM. In this instance, HLM preparations (0.05 mg/ml) were coincubated with solvent alone (acetonitrile, 1.0%, v/v), sulfaphenazole (5 or 10 μM), or montelukast (0.2 or 0.4 μM). The two compounds served as putative inhibitors of CYP2C9 and CYP2C8, respectively. (S)-(+)-IBU similarly incubated in the presence of control mAbs (anti-hen egg white lysozyme, anti-CYP2C8 mAbs, or anti-CYP2C9 mAbs (5 μl of diluted ascites fluid per incubation). The 2-hydroxylation (2-OH) (A) and 3-hydroxylation (3-OH) (B) of (S)-(+)-IBU was determined. Data are presented as the mean (±S.D.) of three determinations and are expressed as percentage inhibition versus solvent alone (chemical inhibitors) or egg white lysozyme mAb (anti-CYP2C9 and anti-CYP2C8 mAbs). See Materials and Methods.
Mouse ascites fluid containing mAbs (anti-CYP2C9, anti-CYP2C8, or anti-hen egg white lysozyme) was diluted with assay buffer and then added to HLMs as described by Krausz et al. (2001). The effect of each mAb preparation on the rates of IBU enantiomer (1, 20, or 500 μM) hydroxylation was then determined using the assay conditions described above. All inhibition data are represented as percent inhibition (versus solvent alone control or control mAb) (eq. 3):

$$\text{% inhibition} = \left[ \frac{v_o - v_i}{v_o} \right] \times 100$$

where $v_o$ is the rate of reaction in the presence of solvent only (or control mAb) and $v_i$ is the rate of reaction in the presence of the chemical inhibitor (or anti-P450 mAb).

### Results

**Kinetics of IBU Hydroxylation.** In the present study, a sensitive LC-MS/MS method was developed to measure the 2- and 3-hydroxylation of (S)-(−)-IBU and (R)-(−)-IBU after incubation with HLMs and recombinant P450s. It was possible to measure metabolite formation under linear conditions (incubation time and protein concentration) and determine $K_m$ and $V_{max}$. Overall, the kinetic parameters shown in Table 1 were found to be more or less similar to those reported by Hamman et al. (1997). Except for the 3-hydroxylation of (R)-(−)-IBU catalyzed by HLMs ($K_m \approx 120 \, \mu M$), and 2-hydroxylation catalyzed by rCYP2C8 ($K_m \approx 280 \, \mu M$), the data were described by two-$K_m$ Michaelis-Menten kinetics, and there was no evidence for autoactivation or substrate inhibition (Korzekwa et al., 1998; Shou et al., 1999; Tracy and Hummel, 2004). In the presence of HLMs, formation of 3-OH was favored over that of 2-OH (CL$_{int}$/CL$_{int}$ ratio $\approx 3$), which is expected because the recovery ratio [(3-OH + 3-carboxy-IBU)/2-OH] of (S)-(−)-IBU and (R)-(−)-IBU metabolites in human excreta is greater than unity (Rudy et al., 1991; Davies, 1998; Rodrigues, 2005). For rCYP2C8, the rates of IBU 3-hydroxylation were too low to permit kinetic analyses.

**Metabolism of IBU Enantiomers in the Presence of Recombinant P450s.** IBU enantiomers (1 μM) were also incubated with a panel of recombinant human P450s and appreciable rates of 2- and 3-hydroxylation were measured only with rCYP2C9 [(S)-(−)-IBU, 0.15 and 0.32 pmol/min/pmol of P450 and (R)-(−)-IBU, 0.11 and 0.17 pmol/min/pmol of P450, respectively]. At a higher concentration of each IBU enantiomer (500 μM), additional P450s were shown to catalyze 3-hydroxylation (CYP2C19) and 2-hydroxylation (CYP2C8, CYP3A4, CYP2C19, CYP2E1, and CYP2B6) (Fig. 2). For both enantiomers, rCYP2C9 demonstrated less regioselectivity (2-OH versus 3-OH formation) compared with other P450s such as rCYP2C8, rCYP3A4, and rCYP2C19. Normalization of turnover, in terms of average P450 isoyme expression in HLMs, confirmed that
The oxidative metabolism of IBU is complex because of the regio- and stereoselective hydroxylation of its enantiomers (Rudy et al., 1991; Hamman et al., 1997; Davies, 1998). However, such hydroxylation has to be viewed in light of additional clearance pathways (Fig. 1). For example, it has been estimated that an appreciable fraction of (R)-(-)-IBU (~60%) metabolism involves unidirectional chiral inversion to the (S)-(+)enantiomer (Davies, 1998). Metabolism via
FIG. 5. Effect of sulfaphenazole and montelukast on IBU enantiomer 2- and 3-hydroxylation catalyzed by HLM preparations from individual organ donors. (R)-(-)-IBU (A) and (S)-(+) IBU (B) (500 μM) were individually incubated with HLMs (0.05 mg/ml) in the presence of sulfaphenazole (10 μM) or montelukast (0.4 μM). For each HLM preparation, data are reported as percentage inhibition (versus solvent alone) and represent the mean of duplicate determinations. Duplicates did not vary by more than 10%. For each HLM preparation, control activities (solvent alone) and genotype are presented in Table 2. The mean (±S.D.) inhibition for each reaction (across all 12 HLM preparations in the panel) is shown.
direct glucuronidation (9%), in addition to 2-hydroxylation (10%) and 3-hydroxylation (20%), has been reported (Rudy et al., 1991; Davies, 1998). In contrast, the (S)-(+-)enantomer of IBU undergoes no detectable chiral inversion and undergoes direct glucuronidation (14%), 2-hydroxylation (28%), and 3-hydroxylation (45%) (Rudy et al., 1991; Davies, 1998; Rodrigues, 2005). Therefore, P450-mediated clearance is more important for the (S)-(+-)enantimer (~70% versus ~30%).

Although the in vitro P450-dependent metabolism of racemic IBU (and its enantiomers) has been documented, the contributions of individual P450s (beyond CYP2C9) in HLM preparations have not been described previously (Leemann et al., 1993; Hamman et al., 1997; McGinnity et al., 2000). Therefore, additional studies were warranted, given the number of reports describing IBU PK-P450 inhibitor combinations and IBU PK-P450 genotype associations (Kirchheiner et al., 2002; Garcia-Martin et al., 2004; Martinez et al., 2005; Hynninen et al., 2006; Bell et al., 2007; Tornio et al., 2007). To this end, an LC-MS/MS assay was developed to monitor the formation of both 2-OH and 3-OH in vitro. The assay enabled assessment of metabolite formation in the presence of HLMs and recombinant P450s under linear conditions. It was possible to evaluate the kinetics of IBU enantiomer hydroxylation, determine $K_m$ and $V_{max}$ for HLMs, rCYP2C9, and rCYP2C8, and confirm the biphasic (two-$K_m$) kinetics described by Hamman et al. (1997). Such a profile may result from two populations of P450s (low $K_m$ and high $K_m$) or the binding of substrate to two sites on a single (dominant) P450 (Korzekwa et al., 1998; Tracy and Hummel, 2004). CYP2C9 is a candidate for the latter scenario, because the enzyme in recombinant form is known to exhibit biphasic kinetics (Hamman et al., 1997; Tracy et al., 2002). This was confirmed with rCYP2C9 (Table 1).

It has been reported that CYP2C9 is a major hydroxylase when HLMs are incubated with racemic IBU and its individual enantiomers (Leemann et al., 1993; Hamman et al., 1997). The data described herein are consistent with CYP2C9 playing a major role in 2-OH and 3-OH formation at low concentrations of IBU (<20 μM). However, CYP2C8 is a second CYP2C subfamily member that has been associated with IBU hydroxylation in vitro, although its exact contribution in HLMs has not been reported (Hamman et al., 1997). Moreover, in vitro P450 reaction phenotype data for both IBU enantiomers over a wide substrate concentration range are not available. Such information is important and may help reconcile the results of clinical drug interaction studies involving IBU enantiomers. For example, gemfibrozil, a known CYP2C8 inhibitor (Ogilvie et al., 2006, and references therein), has been shown to increase the AUC of (R)-(+-)IBU up to 1.6-fold (mean, 1.3-fold) in human subjects (Tornio et al., 2007). In the same study, the effect on the AUC of the IBU (S)-(+-)enantiomer was not statistically significant. Conversely, flucuronazole (CYP2C9 inhibitor) increases (S)-(+-)IBU AUC up to 1.9-fold, while having no effect on the AUC of (R)-(+-)IBU (Hynninen et al., 2006). On the basis of such data, one could argue that CYP2C8 and CYP2C9 play a significant role in the clearance of (R)-(+-)IBU and (S)-(+-)IBU, respectively. This conclusion is consistent with the results of Kirchheiner et al. (2002). These authors were able to show that the AUC of (S)-(+-)IBU was increased (1.7-fold) in subjects genotyped $CYP2C9*3/3$ (versus $CYP2C9*1/3$) with no effect on (R)-(+-)IBU AUC. The impact of CYP2C8 genotype (e.g., CYP2C8*1/3 and CYP2C8*2/3) on IBU enantiomer PK is more difficult to interpret (Garcia-Martin et al., 2005; Martinez et al., 2005), because of the linkage between the CYP2C8*3 and CYP2C9*2 alleles (Rodrigues, 2005, and references therein). CYP2C8 phenotype-genotype associations are further complicated by haplotype (Kirchheiner et al., 2006; Parikh et al., 2007; Rodriguez-Antona et al., 2007; Saito et al., 2007; Tornio et al., 2008). This contrasts with CYP2C9 genotype, which is clearly linked to reductions in drug clearance (CYP2C9*3 > CYP2C9*2) and can be "phenocopied" with inhibitors such as sulfaphenazole and flucuronazole (Kirchheiner and Brockmoller, 2005; Rodrigues, 2005; Gardiner and Begg, 2006).

Despite clinical reports linking CYP2C8 to IBU enantiomer metabolism, in particular (R)-(+-)IBU (Garcia-Martin et al., 2004; Martinez et al., 2005; Tornio et al., 2007), the data presented herein demonstrate that CYP2C8 plays a relatively minor role in the hydroxylation of either enantiomer. First, minimal hydroxylation of both enantiomers was detected with rCYP2C8 (versus rCYP2C9) at the low substrate concentration (1 μM). Even at the higher IBU concentration of 500 μM, the normalized turnover rates measured with recombinant P450s suggested that the contribution of CYP2C8 was minimal (~11%) (Fig. 2). Second, montelukast and anti-CYP2C8 mAbs were relatively weak (~35%) inhibitors of (R)-(+-)IBU and (S)-(+-)IBU hydroxylation in pooled HLMs (Figs. 3 and 4) and individual HLM (Fig. 5) preparations. However, despite such weak inhibition, it was possible to discern some preference for 2-hydroxylation (11–24% inhibition in pooled HLMs and 14–35% inhibition for individual HLMs) over 3-hydroxylation (3–8% inhibition in pooled HLMs and 5–28% inhibition for individual HLMs). Although additional P450s (e.g., CYP3A4 and CYP2C19) catalyze IBU hydroxylation in HLMs, the above observation is consistent with the regioelective hydroxylation (2-OH/3-OH ratio ~10) observed with rCYP2C8 (Fig. 2).

Taken together, the P450 reaction phenotype data described herein indicated that the respective contributions of CYP2C9, CYP2C8, CYP3A4, and CYP2C19 in HLMs are ~45, ~23, 33, and 9% [2-hydroxylation of (S)-(+-)IBU], ~80, ~14, 11, and ~5% [3-hydroxylation of (S)-(+-)IBU], ~50, ~25, 32, and 5% [2-hydroxylation of (R)-(+-)IBU], and ~85, ~17, 14, and ~<2% [3-hydroxylation of (R)-(+-)IBU] at a high concentration of IBU (500 μM). Given the known P450 selectivity of sulfaphenazole and the anti-CYP2C9 mAb preparations used, it can be assumed that CYP2C9 plays a major role (75–100%) in both hydroxylation reactions at lower concentrations of IBU (~20 μM). At the same low IBU concentrations, the contribution of CYP2C8 is low (~25%).

When all IBU enantiomer clearance pathways are considered (direct glucuronidation to the acyl glucuronide, chiral inversion, 2-hydroxylation, and 3-hydroxylation) and the present reaction phenotype data are integrated across all concentrations of IBU tested, it is calculated that CYP2C9 accounts for 49 to ~70% [(S)-(+-)IBU] and 20 to 30% [(R)-(+-)IBU] of total clearance. This result is consistent with the reported impact of the CYP2C9*3/3 genotype and flucuronazole on (S)-(+-)IBU AUC (versus (R)-(+-)IBU AUC) (Kirchheiner et al., 2002; Rodrigues, 2005; Hynninen et al., 2006). On the other hand, CYP2C8 contributes to only a small fraction of (R)-(+-)IBU (~6%) and (S)-(+-)IBU (~13%) clearance. Therefore, (R)-(+-)IBU AUC increases of greater than 2-fold in subjects carrying mutant CYP2C8 alleles are difficult to rationalize on the basis of the in vitro data presented herein and published ADME information (Garcia-Martin et al., 2004; Martinez et al., 2005; Rodrigues, 2005). The increased (R)-(+-)IBU AUC (up to 1.6-fold) with gemfibrozil, in the absence of an effect on (S)-(+-)IBU AUC, is also difficult to reconcile with the present data and requires further investigation (Tornio et al., 2007). It is possible that gemfibrozil, a known OATP inhibitor, inhibits the hepatic uptake of IBU enantiomers [(R)-(+-)IBU > (S)-(+-)IBU]. However, this possibility appears unlikely because neither enantiomer (1–500 μM) served as a substrate of human OATP1B1, OATP1B3, and OATP2B1 in vitro (data not shown). Chiral inversion of (R)-(+-)IBU to (S)-(+-)IBU is an enzyme-cata-
lyzed process, and inhibition by gemfibrozil also remains a possibility (Hao et al., 2005). Then again, no inhibition of (R)-(−)-IBU chiral inversion was evident in suspensions of primary human hepatocytes cocultivated with gemfibrozil (2–200 μM) (data not shown).

Going forward, the CYP2C8-dependent metabolism of IBU enantiomers has to be considered in light of all P450 and non-P450 clearance pathways and the possible involvement of additional P450s beyond CYP2C8 and CYP2C9 (e.g., CYP2C19 and CYP3A4). In particular, changes in (R)-(−)-IBU AUC cannot be ascribed solely to CYP2C8 without regard for possible impact on chiral inversion. Furthermore, one has to take into account the complexities of the CYP2C8 genotype-phenotype relationship. For example, compared with IBU enantiomers, CYP2C8 plays a significant role in the in vitro metabolism of paclitaxel, rosiglitazone, and pioglitazone. Despite a prominent role in vitro, the impact of CYP2C8 genotype (e.g., CYP2C8*3 allele) on the PK of most of the above drugs is not clear (Kirchheiner et al., 2006; Parikh et al., 2007; Rodríguez-Antonio et al., 2007; Saito et al., 2007; Tornio et al., 2008). Therefore, the metabolic profile of IBU enantiomers (after dosing of subjects carrying different CYP2C9 and CYP2C8 alleles) requires further study, and additional radiolabeled studies may be warranted.

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