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Regioselective and Stereoselective Metabolism of Ibuprofen by Human Cytochrome P450 2C

Mitchell A. Hamman, Gary A. Thompson† and Stephen D. Hall*‡*

*Division of Clinical Pharmacology, Indiana University School of Medicine, Indianapolis, IN 46202, U.S.A.; and †Clinical Pharmacology Department, Procter & Gamble Co., Cincinnati, OH 45242, U.S.A.

ABSTRACT. The cytochrome P450s responsible for the regio- and stereoselectivity in the 2- and 3-hydroxylation of the chiral non-steroidal antiinflammatory drug ibuprofen were characterized in human liver microsomes. The rates of formation of both the 2- and 3-hydroxy metabolites exhibited monophasic (N = 2; N is the number of microsomal preparations) and biphasic (N = 2) substrate concentration dependence for both enantiomers of ibuprofen. The high affinity enzyme class parameters for *S*-ibuprofen (N = 4) were: 2-hydroxylation, V_{max} = 566 ± 213 pmol/min/mg, K_m = 38 ± 13 μM; 3-hydroxylation, V_{max} = 892 ± 630 pmol/min/mg, K_m = 21 ± 6 μM. For *R*-ibuprofen, the corresponding parameters were: 2-hydroxylation, V_{max} = 510 ± 117 pmol/min/mg, K_m = 47 ± 20 μM; 3-hydroxylation, V_{max} = 593 ± 113 pmol/min/mg, K_m = 29 ± 8 μM. cDNA-expressed CYP2C9 (Arg 144 and Cys 144) favored *S*-2- and *S*-3-hydroxyibuprofen formation, but CYP2C8 favored *R*-2-hydroxyibuprofen formation. Sulfaphenazole, retinol, and arachidonic acid competitively inhibited the rate of formation of all hydroxyibuprofens; K_i values (N = 3) for sulfaphenazole on the 2- and 3-hydroxylations of *S*-ibuprofen were 0.12 ± 0.05 and 0.07 ± 0.04 and of *R*-ibuprofen were 0.11 ± 0.07 and 0.06 ± 0.03 μM, respectively. Sulfaphenazole also competitively inhibited ibuprofen hydroxylation by cDNA-expressed CYP2C9 (Arg 144 and Cys 144) with K_i values in the range of 0.05 to 0.18 μM and CYP2C8 in the range of 0.36 to 0.55 μM. In a bank of 14 human liver microsome samples, significant correlations (r = 0.72 to 0.90; $P < 0.01$) were observed between the rates of formation of all four hydroxyibuprofens, and for each hydroxyibuprofen and prototypical CYP2C8/9 biotransformations. The regio- and stereoselectivities observed *in vitro* were consistent with those noted *in vivo*. The relative levels of both CYP2C8 and CYP2C9 and the expression of the corresponding variants may influence the disposition of ibuprofen *in vivo*. BIOCHEM PHARMACOL **54**;1:33–41, 1997. © 1997 Elsevier Science Inc.

KEY WORDS. ibuprofen; enantiomers; cytochrome P450; microsomal oxidation; human biotransformation

Ibuprofen is an NSAID§ commonly administered for its antiinflammatory, analgesic, and antipyretic properties. As a member of the 2-arylpropionic acid class of NSAIDs, ibuprofen contains a chiral center and is employed therapeutically as a racemic mixture. Following oral administration of racemic ibuprofen, approximately 60% of the dose is recovered in a 24-hr urine collection as 2-OHIbu, 3-carboxyibuprofen, and their conjugates (Fig. 1; [1–3]). The formation clearances associated with these metabolites *in vivo* suggest a distinct stereoselectivity in favor of the *S*-enantiomer, and regioselectivity in favor of 3-carboxyibuprofen formation [2, 3]. Preliminary studies have been used to suggest that only CYP2C9 is involved in the oxidation of ibuprofen [4]. However, the involvement of a single enzyme is inconsistent with the dramatic and stereoselective increases in plasma concentrations of *S*-ibuprofen and increased risk of morbidity in some, but not all, patients with moderate renal impairment [3, 5].

In common with the other members of the "profen" class of NSAIDs, the *S*-enantiomer of ibuprofen is responsible primarily for the inhibition of cyclooxygenase and, therefore, prostaglandin synthesis [6]. This property has been considered traditionally as the sole mechanism of action of the profens and NSAIDs in general [7]. However, it is becoming increasingly apparent that actions in addition to cyclooxygenase inhibition may contribute to pharmacological profiles of individual NSAIDs [8, 9]. Members of the human CYP2C subfamily, CYP2C8 and 2C9 in particular, clearly are involved in the oxidation of unsaturated fatty acids to active metabolites [10, 11]. For example, CYP2C8 and 2C9 stereoselectively epoxidized arachidonic acid to 14(*R*), 15(*S*)-EET but showed opposite stereoselectivities in the formation of 11,12-EET [10]. The stereoselective formation of 11(*R*), 12(*S*)-EET by CYP2C8 corresponded to the products of arachidonic acid oxidation by human kidney cortex, suggesting that CYP2C8 is the dominant EET-producing enzyme in some cell types and may, there-

‡ Corresponding author: Stephen D. Hall, Ph.D., Clinical Pharmacology Section, 320 OPW Wishard Memorial Hospital, 1001 West Tenth St., Indianapolis, IN 46202-2879. Tel. (317) 630-8795; FAX (317) 630-8185.

§ *Abbreviations:* CYP, cytochrome P450; EET, epoxyeicosatrienoic acid; NSAID, non-steroidal antiinflammatory drug; 2-OHIbu, 2-hydroxyibuprofen; and 3-OHIbu, 3-hydroxyibuprofen.

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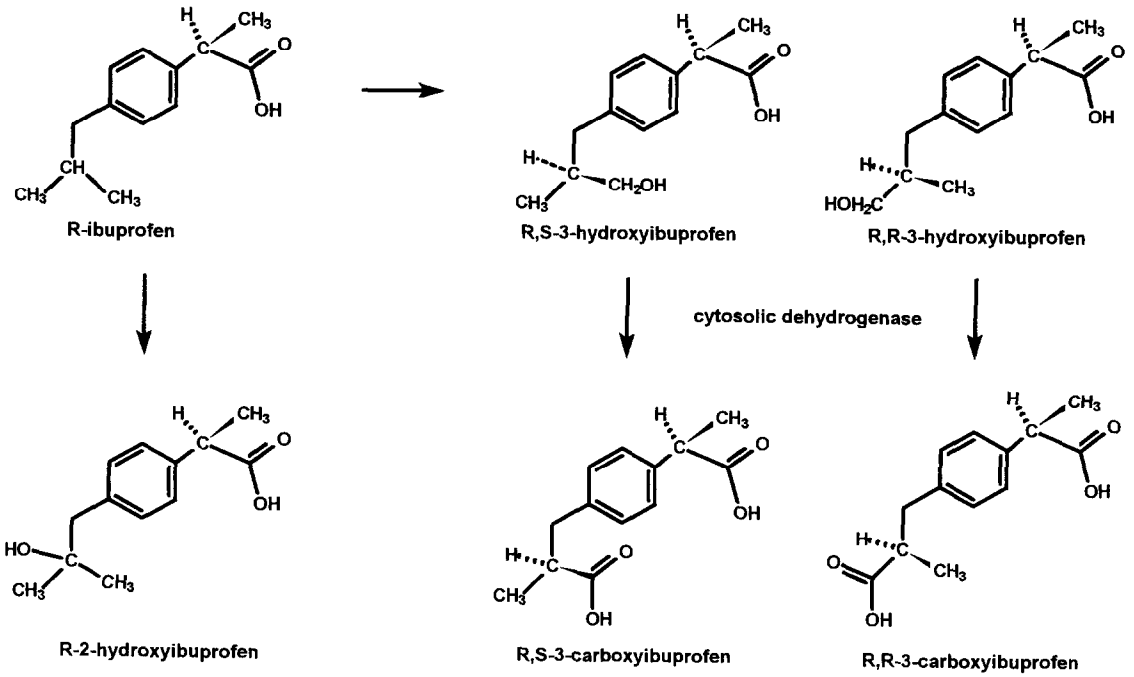

FIG. 1. Oxidative metabolism of the enantiomers of ibuprofen in humans. When the substrate is *R*-ibuprofen, cytochrome P450 produces *R*-2-OHIbu and two diastereomeric 3-OHIbus designated *R,S*- and *R,R*-3-OHIbu. For *S*-ibuprofen, the corresponding products are *S*-2-OHIbu *S,S*-3-OHIbu and *S,R*-OHIbu. *In vivo*, the 3-OHIbus undergo nonmicrosomal dehydrogenation to the corresponding 3-carboxyibuprofens.

fore, play a critical role in the regulation of integrated organ function [10–12]. Therefore, the potential exists for interactions between NSAIDs, such as ibuprofen, and EET formation via inhibition of CYP2C enzymes, which in turn may contribute to the pharmacological profile of these widely used drugs. This phenomenon would be analogous to the interactions of ibuprofen with other drug substrates of CYP2C9, such as coumarins and hydantoins. In general, the relative contribution of enzymes in the CYP2C subfamily and other CYPs to the oxidative metabolic clearance of ibuprofen is unclear. Therefore, as a first step in predicting the effects of disease states on ibuprofen clearance and the effect of ibuprofen on the biotransformation of both endo- and xenobiotics, we have characterized the human microsomal metabolism of *R*- and *S*-ibuprofen in detail.

MATERIALS AND METHODS

Chemicals

R(−)- and S(+)-ibuprofen were obtained from Sepracor, Inc. (Marlborough, MA), with a chemical purity of 99.5% and optical purity of 99 and 99.9%, respectively. 2-[4′-(2-Hydroxy-2-methylpropyl)phenyl]propionic acid (2-OHIbu) was supplied by the Upjohn Co. (Kalamazoo, MI). Racemic ketoprofen, diethyldithiocarbamic acid, all-*trans* retinol (80% purity), arachidonic acid, quinidine, lauric acid, 12-hydroxylauric acid, caffeine, 1,7-dimethylxanthine, troleandomycin, and coumarin were purchased from the Sigma Chemical Co. (St. Louis, MO). Sulfaphenazole was obtained from Ciba–Geigy (Sydney, Australia). Furafylline was acquired from Research Biochemicals International (Natick, MA). S-Mephenytoin was obtained from Ultrafine Chemicals (Manchester, U.K.). Microsomes from B-lymphoblastoid cell lines expressing CYP1A2, CYP2A6, CYP2C8, CYP2C9 (Arg 144 and Cys 144), CYP2C19, CYP2D6, CYP2E1, CYP3A4, CYP4A11, and control microsomes were purchased from Gentest (Woburn, MA). All other chemicals were purchased from commercial sources, and were of the highest purities available.

Microsomes

A bank of 14 liver samples (designated HL-A through HL-N) was obtained under protocols approved by the Medical College of Wisconsin (Madison, WI) and were provided by Dr. S. A. Wrighton, Eli Lilly & Co. (Indianapolis, IN; [13]). Two additional liver samples, designated IU3 and IU11, were obtained under a protocol approved by the Institutional Review Board of the Indiana University Medical Center. Microsomes were prepared by differential centrifugation as described previously [14]. The microsomes were stored in a 100 mM potassium phosphate buffer, pH 7.4, containing 1 mM EDTA, 20% glycerol, 20 μM butylated hydroxytoluene, 100 μM phenylmethylsulfonyl fluoride, and stored at −70°C until used. The protein concentrations were measured by the method of Lowry *et al.* [15].

Microsomal Incubations

Microsomal incubations contained sodium phosphate buffer (100 mM, pH 7.4, 0.1 mM EDTA) and 0.2 mg liver

microsomal protein or 1.0 mg B-lymphoblastoid microsomal protein. *R*- or *S*-ibuprofen was added in 2.5 μL of methanol, and the mixture was preincubated at 37° for 3 min. The reaction was initiated by the addition of 1 μmol NADPH to give a final volume of 1 mL and was allowed to continue at 37° for 20 min for liver microsomes or for 1 hr for B-lymphoblastoid microsomes. Substrate concentration dependence was determined at 0, 5, 10, 25, 50, 100, 250, 500, and 1000 μM *R*- and *S*-ibuprofen. Preliminary experiments verified that initial rate conditions were maintained under these incubation conditions. Putative inhibitors were preincubated along with substrate for 3 min with the exception of troleandomycin and furaphylline, which were preincubated with NADPH for 20 min prior to reaction initiation with *R*- or *S*-ibuprofen. All incubations were terminated with the addition of 1 mL ethyl acetate: hexane (85:15, v/v) and placed on ice.

HPLC Analysis of Hydroxyibuprofen

The concentrations of 2-OHIbu and 3-OHIbu (2-[4'-(3-hydroxy-2-methylpropyl)phenyl]propionic acid) were determined by reverse phase chromatography with a step gradient elution. Briefly, 50 μL of racemic ketoprofen (20 ng/mL; internal standard) and 50 μL of 50% phosphoric acid were added to the microsomal incubation, and the mixture was extracted with 3.5 mL of ethyl acetate:hexane (85:15, v/v). After centrifugation at 2000 *g* for 10 min, the organic layer was removed and evaporated to dryness and reconstituted with 100 μL of mobile phase A (see below), from which 20–50 μL was injected. Chromatographic separation was achieved with a 5 μm octyl column (100 × 4.6 mm i.d.; Applied Biosystems, Foster City, CA). Mobile phase A (50 mM potassium phosphate, pH 3:acetonitrile; 67:33, v/v) was eluted isocratically at 0.5 mL/min for 7 min, and then stepped to 100% mobile phase B (50 mM potassium phosphate, pH 3:acetonitrile; 47:53, v/v) for an additional 13 min, and monitored by ultraviolet absorbance at 230 nm. The retention times for 2-OHIbu, 3-OHIbu, and ketoprofen (internal standard) were 6, 7.3, and 16 min, respectively. Interday coefficient of variation for 2-OHIbu at 500 pmol injected was 6.8%. An authentic 3-OHIbu standard was unavailable. The chromatographic peak presumed to be 3-OHIbu was collected, and a trimethylsilyl derivative was prepared for identification by gas chromatography mass spectrometry [16]. The 3-OHIbu derivative was eluted with helium, as carrier gas, and a thermal gradient on an HP-1, 30 m capillary column (Hewlett Packard, Palo Alto, CA). A 70 eV electron impact mass spectrum was obtained with an HP5971A mass selective detector (Hewlett Packard). The spectrum was consistent with a published fragmentation pattern for derivatized 3-OHIbu [16]. The response factors and extraction efficiencies for 2-OHIbu and 3-OHIbu were presumed equal.

The 3-hydroxylation of *R*- or *S*-ibuprofen introduces a second chiral center and, therefore, a pair of diastereomeric 3-hydroxy metabolites are expected for each optically pure substrate (Fig. 1). However, there was no apparent resolution of these diastereomers using reverse phase or chiral HPLC. In view of the absence of authentic standards, no attempt was made to resolve the diastereomers following derivatization. 2-OHIbu and 3-OHIbu were assumed to have the same configuration, at the 2-position of the propionic acid moiety, as the initial optically pure substrate, that is, *R*- or *S*-ibuprofen. This assumption is reasonable given that (1) chiral inversion of ibuprofen does not occur *in vitro* in the absence of coenzyme A, and (2) following the administration of the individual enantiomers to humans, the stereochemical composition of the metabolites derived from 2- and 3-hydroxylation reflects the chiral inversion of *R*- and *S*-ibuprofen [1–3]. Therefore, inversion of the metabolites of ibuprofen does not appear to occur in humans.

Other HPLC Analysis

6α-Hydroxytaxol derived from taxol was assayed by Dr. A. Rahman (U.S. Food and Drug Administration, Rockville, MD) using reverse phase chromatography with gradient elution described previously [17]. The determination of 1,7-dimethylxanthine formation from caffeine was measured by gradient reverse phase with ultraviolet absorbance detection by the method of Tassaneeyakul *et al.* [18]. The fluorimetric measurement of the coumarin derivative of ω-hydroxylauric acid was assayed by the method of Dirven *et al.* [19].

Data Analysis

Metabolite formation data are the means of duplicate determinations for each condition. Substrate concentration, [S], and dependence of metabolite formation rate, (v), initially were examined for single or multiple enzyme kinetics by the linearity of Eadie-Hofstee plots [20] (v vs v/[S]). The kinetic parameters were determined by nonlinear regression (PCNONLIN version 4.0; SCI Software, Lexington, KY), and fitted to either equation 1:

$$v = \frac{V_{max} \cdot [S]}{K_m + [S]} \quad (1)$$

or equation 2:

$$v = \frac{V_{max1} \cdot [S]}{K_{m1} + [S]} + CL_{int2} \cdot [S] \quad (2)$$

where V_{max} is the maximal rate of metabolite formation, and K_m is the substrate concentration at half of the maximal velocity. The parameter CL_{int2} is the ratio of V_{max2}/K_{m2} for the low affinity, unsaturated component of a two-enzyme system. For the single-enzyme equation, a weight of 1/[S], and for the two-enzyme equation unweighted data, was found to be most appropriate for nonlinear regression. The choice of the most appropriate

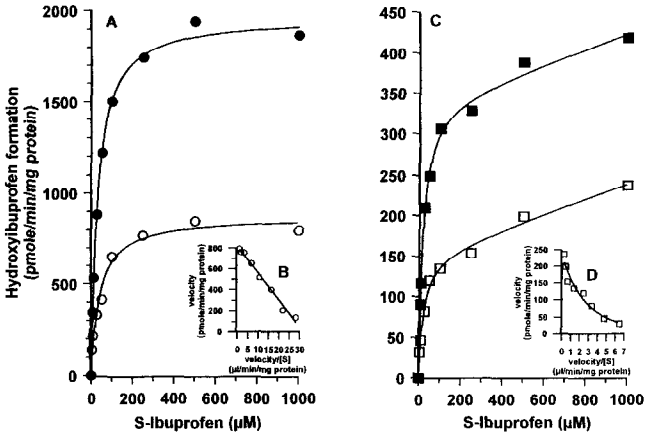

FIG. 2. (A and C) Dependence of the rate of 2-hydroxylation (open symbols) and 3-hydroxylation (closed symbols) on S-ibuprofen concentration for microsomes from human liver IU3 (circles) and HL-K (squares). Lines of best fit to equations 1 or 2, as appropriate, were determined by nonlinear regression of untransformed data (see text). (B and D) Corresponding Eadie–Hofstee plots.

kinetic model was determined from an inspection of residuals, residual sums of squares (partial F-test), the magnitude of the standard error in the parameter estimates and, for non-hierarchical models, the Akaike information criterion [21]. Competitive inhibition was modeled by [20]:

$$v = \frac{V_{max} \cdot [S]}{K_m \cdot (1 + \frac{[I]}{K_i}) + [S]} \quad (3)$$

where K_i is the equilibrium inhibition constant and (I) the inhibitor concentration. A weighting of 1/[S] was again found to be most appropriate. Confirmation of the appropriateness of a competitive model was obtained from the appearance of Dixon plots [20]. Group mean parameter estimates were compared using analysis of variance followed by the Student–Newman–Keuls multiple range test, and correlation coefficients with corresponding level of statistical significance were calculated by conventional methods (PC-SAS; SAS Institute Inc., Carey, NC).

RESULTS

The production of 2- or 3-OHIbu from *R*- and *S*-ibuprofen was linear for 45 min with a substrate concentration of 100 μM and 0.2 mg microsomal protein. Michaelis–Menten parameters were estimated for four human liver microsomal preparations. For two microsomal preparations, the linear appearance of the Eadie–Hofstee plots and the good fit of the one class enzyme model (equation 1) suggested a single enzyme or multiple enzymes with similar K_m values for the formation of all four hydroxyibuprofens (Fig. 2). In contrast, two microsomal preparations exhibited biphasic Eadie–Hofstee plots, and were characterized by a model that included an additional low affinity, unsaturated enzyme class (equation 2; Fig. 2). A model specifying two saturable enzyme classes resulted in poor parameter estimates and large residual sums of squares. The model parameter estimates for the formation of 2- and 3-OHIbu derived from either *R*- or *S*-ibuprofen are shown in Table 1. The intrinsic clearance of the high affinity enzyme was highest for *S*-3-OHIbu followed by *R*-3-OHIbu > *S*-2-OHIbu > *R*-2-OHIbu. The microsomes that displayed biphasic kinetics showed an intrinsic clearance order for

TABLE 1. Parameters describing the substrate concentration dependence of the rates of 2- and 3-hydroxylation of *R*- and *S*-ibuprofen in four human liver microsomal preparations

	2-Hydroxylation				3-Hydroxylation			
Liver I.D.	V_{max1} (pmol/min/mg protein)	K_{m1} (μM)	CL_{int1} (μL/min)	CL_{int2} (μL/min)	V_{max1} (pmol/min/mg protein)	K_{m1} (μM)	CL_{int1} (μL/min)	CL_{int2} (μL/min)
				R-Ibuprofen				
HLE	690 ± 16*	73 ± 4	9.5	†	543 ± 19	26 ± 3	20.6	†
IU3	512 ± 8	44 ± 2	11.7	†	788 ± 14	42 ± 2	19.1	†
HLK	364 ± 18	17 ± 2	21.4	0.15 ± 0.03	531 ± 31	18 ± 3	29.5	0.16 ± 0.05
IU11	475 ± 69	54 ± 16	8.8	0.28 ± 0.06	509 ± 23	29 ± 3	17.4	0.21 ± 0.02
Mean	510	47	12.9	0.22	593	29	21.7	0.19
SD	117	20	5.1		113	8	4.7	
				S-Ibuprofen				
HLE	508 ± 23	48 ± 9	10.6	†	625 ± 18	21 ± 2	29.5	†
IU3	842 ± 42	34 ± 5	25	†	1961 ± 91	31 ± 4	62.9	†
HLK	260 ± 16	19 ± 3	14.1	0.13 ± 0.02	332 ± 17	16 ± 2	20.6	0.10 ± 0.03
IU11	644 ± 151	50 ± 24	13.1	0.59 ± 0.13	651 ± 46	16 ± 3	40	0.40 ± 0.05
Mean	566	38	15.8	0.36	892	21	38.3	0.25
SD	213	13	5.6		630	6	15.8	

Parameter estimates were obtained using equations 1 or 2 (see text). Data represent the means of duplicate determinations.

* Parameter estimates are reported ± SE; standard error represents the asymptotic error of the parameter estimated by nonlinear regression.

† Model describes one class of enzyme.

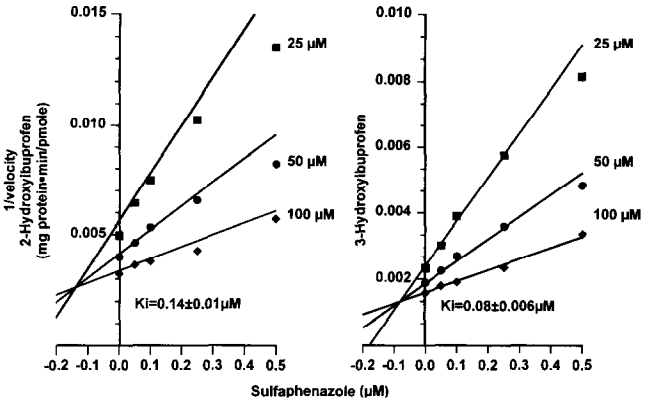

FIG. 3. Dixon plots illustrating the competitive inhibition of the 2- and 3-hydroxylations of *S*-ibuprofen by sulfaphenazole in microsomes from human liver IU11. Lines of best fit were determined by nonlinear regression using untransformed data.

the low affinity enzyme *S*-2-OHIbu > *S*-3-OHIbu > *R*-2-OHIbu > *R*-3-OHIbu.

Selective inhibitors of CYP1A2, CYP2A6, CYP2C19, CYP2D6, CYP2E1, and CYP3A4/5 were utilized to assess the potential contribution of these enzymes to ibuprofen hydroxylation in a microsomal preparation (IU11) displaying a biphasic Eadie–Hofstee plot. The inhibitors were incubated at two or more concentrations with 100 μM *R*- or *S*-ibuprofen and compared with a vehicle only control. Furafylline (CYP1A2; 1 and 5 μM), coumarin (CYP2A6; 100 and 1000 μM), S-mephenytoin (CYP2C19; 5 and 20 μM), quinidine (CYP2D6; 1 and 5 μM), diethyldithiocarbamic acid (CYP2E1; 50 and 300 μM), and troleandomycin (CYP3A4/5; 100 and 300 μM) decreased the 2- and 3-hydroxylation rates by less than 5%, compared with the control.

Sulfaphenazole was a potent, competitive inhibitor of ibuprofen oxidation, and inhibition was regioselective in favor of the 3-hydroxylation (Fig. 3). The estimated K_i values (N = 3) suggested that sulfaphenazole was more potent towards 3-OHIbu formation compared with 2-OHIbu formation, but this difference was not statistically significant ($P > 0.05$; Table 2). The K_i values for sulfaphenazole (0.06 to 0.12 μM) were consistent with those reported for other CYP2C9 biotransformations, such as *S*-warfarin 7-hydroxylation [22]. To further explore the determinants of *R*- and *S*-ibuprofen (25, 50, and 100 μM) hydroxylation inhibition by a substrate of CYP2C8, all *trans*-retinol [23] and a substrate for 2C8 and 9 [10], arachidonic acid, were examined at concentrations of 0, 1, 5, 10, and 25 μM. Retinol and arachidonic acid were both potent competitive inhibitors of *R*- and *S*-ibuprofen hydroxylation in liver microsomes and displayed little stereo- or regioselectivity (Table 2).

Microsomes derived from a B-lymphoblastoid cell line expressing CYP1A2, CYP2A6, CYP2C8, CYP2C9 (Arg 144 and Cys 144), CYP2C19, CYP2D6, CYP2E1, CYP3A4, and CYP4A11 were employed to evaluate the potential for these enzymes to oxidize *R*- or *S*-ibuprofen at 100 μM. CYP1A2 and CYP3A4 produced a trace level of 2- and 3-OHIbu but CYP2A6, CYP2C19, CYP2D6, and CYP2E1 failed to produce measurable amounts of hydroxyibuprofen metabolites. CYP2C9 (Arg 144 and Cys 144) and CYP2C8 demonstrated significant ibuprofen hydroxylation and exhibited monophasic Eadie–Hofstee plots when examined in detail (data not shown). CYP2C9 (K_m 35–82 μM) exhibited the greatest intrinsic clearances with selectivity towards *S*-3-OHIbu formation (Fig. 4). CYP2C8 (K_m 3.5–74 μM) and the Cys 144 variant of CYP2C9 (K_m 16–63 μM) were less active than CYP2C9 (Fig. 4). In contrast to CYP2C9 (Arg 144 and Cys 144), CYP2C8 demonstrated significant regioselectivity in favor of 2-OHIbu formation (Fig. 4). Sulfaphenazole competitively

TABLE 2. Equilibrium inhibition constants (K_i) reflecting the competitive inhibition of 2- and 3-hydroxylation of *R*- and *S*-ibuprofen

Liver I.D.	K_i (μM)			
	R-Ibuprofen		*S*-Ibuprofen	
	2-Hydroxylation	3-Hydroxylation	2-Hydroxylation	3-Hydroxylation
		Sulfaphenazole		
IU11	0.13 ± 0.005*	0.08 ± 0.002	0.14 ± 0.01	0.08 ± 0.006
HLD	0.02 ± 0.004	0.014 ± 0.003	0.05 ± 0.005	0.015 ± 0.002
IU3	0.19 ± 0.03	0.09 ± 0.006	0.16 ± 0.007	0.11 ± 0.01
Mean	0.11	0.06	0.12	0.07
SD	0.07	0.03	0.05	0.04
		Retinol		
IU11	5.9 ± 0.8	4.7 ± 0.6	5.5 ± 0.7	4.7 ± 0.8
HLI	8.9 ± 2.2	8.5 ± 1.9	9.5 ± 1.5	6.9 ± 1.4
Mean	7.4	6.6	7.5	5.8
		Arachidonic acid		
IU11	2.6 ± 0.7	1.7 ± 0.5	2.8 ± 0.5	2.2 ± 0.4

Data represent the means of duplicate determinations.

* Parameter estimates are reported ± SE; standard error represents the asymptotic error of the parameter estimated by nonlinear regression.

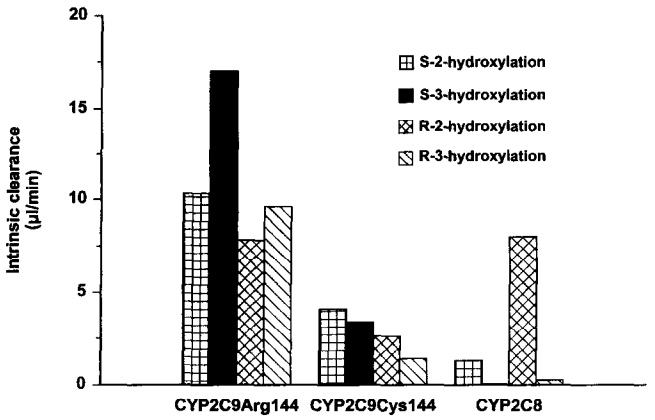

FIG. 4. Comparison of intrinsic clearance values of 2-OHIbu and 3-OHIbu formation by cDNA-expressed CYP2C8, CYP2C9 (Arg 144), and CYP2C9 (Cys 144) variant. Parameter estimates were obtained from the means of duplicate determinations.

inhibited the 2- and 3-hydroxylations of *R*- and *S*-ibuprofen by cDNA-expressed proteins with K_i values of 0.17, 0.16, 0.08, and 0.18 µM, respectively, for CYP2C9 (Arg 144); 0.05, 0.07, 0.05, and 0.06 µM for CYP2C9 (Cys 144), respectively; and 0.42, 0.55, 0.36, and 0.38 µM for CYP2C8, respectively.

Correlations between the rates of *R*- and *S*-ibuprofen (1 mM) hydroxylation and either form selective activities or immunoquantified enzyme levels were examined in a bank of 14 human liver microsomes (HL-A through HL-N) that have been characterized previously [13]. Highly significant ($P \leq 0.01$) correlations were noted between hydroxyibuprofen formation rates and markers of CYP2C8/9 catalytic activity, that is tolbutamide methyl hydroxylase, phenytoin 4′-hydroxylase, *S*-warfarin 7-hydroxylase, and taxol 6α-hydroxylase (Table 3); the intercept of the regression lines was not significantly different from zero. Significant ($P \leq 0.05$) correlations were also noted with dextromethorphan O-demethylase (CYP2D6) and chlorozoxazone 6-hydroxylase (CYP2E1) activities (Table 3), but there was no correlation with immunoquantified levels of CYP2D6 or CYP2E1. *R*-2-OHIbu differed from the other metabolites in that a weak correlation with midazolam 1′-hydroxylase activity and immunoquantified CYP1A2 and CYP2C19 was observed (Table 3). There were significant ($P \leq 0.05$) correlations between 6α-hydroxylation and CYP2C9 activities, such as tolbutamide methyl hydroxylation (r =

TABLE 3. Correlations between *R*- and *S*-ibuprofen hydroxylation and the immunoquantified levels or selective activities of human cytochrome P450s in a bank of 14 liver microsomal samples

Cytochrome P450	*R*-Ibuprofen 2-hydroxylation	*R*-Ibuprofen 3-hydroxylation	*S*-Ibuprofen 2-hydroxylation	*S*-Ibuprofen 3-hydroxylation
CYP1A2				
Immunoquantified levels	0.64*	0.48	0.50	0.46
Caffeine 3-demethylation	0.18	0.08	0.07	0.09
CYP2A6				
Immunoquantified levels	0.32	0.14	0.31	0.12
Coumarin 7-hydroxylation	0.45	0.35	0.45	0.34
CYP2C8				
Immunoquantified levels	0.47	0.22	0.39	0.17
Taxol 6α-hydroxylation	0.76†	0.82†	0.86†	0.79†
CYP2C9				
Immunoquantified levels	0.30	0.27	0.32	0.25
Tolbutamide methylhydroxylation	0.75†	0.83†	0.86†	0.80†
Phenytoin 4′-hydroxylation	0.76†	0.82†	0.90†	0.80†
S-Warfarin 7-hydroxylation	0.72†	0.81†	0.87†	0.78†
CYP2C19				
Immunoquantified levels	0.58*	0.22	0.33	0.13
S-Mephenytoin 4′-hydroxylation	0.51	0.25	0.27	0.17
CYP2D6				
Immunoquantified levels	0.44	0.16	0.28	0.14
Dextromethorphan O-demethylation	0.68†	0.66†	0.59*	0.61*
CYP2E1				
Immunoquantified levels	0.37	0.48	0.51	0.53
Chlorzoxazone 6-hydroxylation	0.37	0.55*	0.56*	0.61*
CYP3A4				
Immunoquantified levels	0.44	0.02	0.21	0.10
Midazolam 1′-hydroxylation	0.60*	0.15	0.35	0.04
CYP4A				
Immunoquantified levels	0.45	0.34	0.46	0.34
Lauric acid ω-hydroxylation	0.02	0.04	0.08	0.07

* $P \leq 0.05$.
† $P \leq 0.01$.

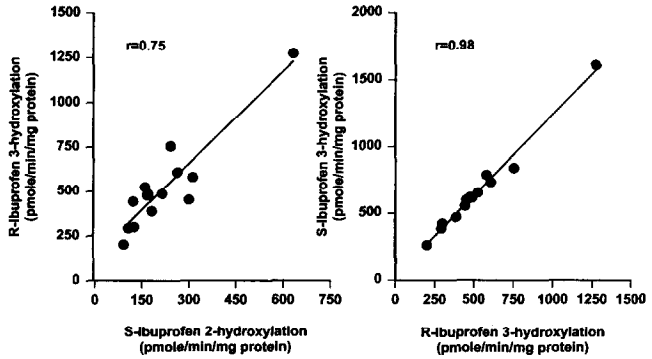

FIG. 5. Correlation between the rates of *R*-ibuprofen 3-hydroxylation and the rates of *S*-ibuprofen 2- and 3-hydroxylation.

0.73). The lack of correlation between immunoquantified CYP2C9 and prototypical catalytic activities has been discussed previously [24].

An all but perfect correlation between the rates of formation of *R*- and *S*-3-OHIbu was noted and a slightly lower correlation between *S*-2-OHIbu and *S*-3-OHIbu was noted (Fig. 5 and Table 4). In contrast, the correlation between rates of formation of *R*- and *S*-2-OHIbu and between *R*-2-OHIbu and *R*-3-OHIbu was not as high, but the least significant correlation was between the rates of formation of *R*-2-OHIbu and *S*-3-OHIbu (Table 4).

DISCUSSION

Ibuprofen is one of the most widely and frequently employed therapeutic agents for the treatment of acute and chronic pain and inflammation. With few exceptions, ibuprofen is available only as a racemic mixture despite the fact that the *S*-enantiomer is primarily responsible for the inhibition of cycloxygenase activity [6]. Administration of the individual enantiomers has revealed that approximately 60% of a dose of *R*-ibuprofen is inverted to *S*-ibuprofen in humans by virtue of its capability to form a coenzyme A conjugate; there is no significant *S*- to *R*-ibuprofen inversion [2, 25, 26]. Conjugation of ibuprofen with glucuronic acid exhibits a significant degree of enantioselectivity in favor of *S*-ibuprofen but constitutes a relatively minor elimination pathway in humans (approximately 10% dose; [2, 25, 26]). The major pathways of ibuprofen elimination in humans are oxidative (approximately 70% dose) and display relatively modest stereoselectivity. Specifically, the unbound formation clearances of 2-OHIbu and 3-carboxyibuprofen exhibit *S*:*R* enantioselectivities of 1.1 and 1.7, respectively [2, 3]. These enantioselectivities are in excellent agreement with the *S*:*R* ratios for *in vitro* intrinsic clearance observed in human liver microsomes in this study, that is, 1.2 and 1.8 for the 2- and 3-hydroxylations, respectively (Table 1). The similar enantioselectivities observed *in vivo* and *in vitro* suggest that secondary metabolism, such as cytosolic carboxylation of 3-OHIbu, does not contribute to the enantioselectivity of ibuprofen elimination *in vivo*. Consequently, human liver microsomes represent a robust system for identifying the enzymes that control the major pathways of ibuprofen elimination.

Several risk factors have been identified that predispose an individual to ibuprofen-induced renal dysfunction [27]. The most important of these factors is pre-existing renal impairment, when the maintenance of tubular perfusion requires prostaglandin synthesis [27, 28]. Chen and Chen [5] suggested that, in a group of Oriental patients from Taiwan, a high risk of ibuprofen-induced renal impairment was associated with increased plasma concentrations of *S*-ibuprofen, but not *R*-ibuprofen. This enantioselective change in plasma concentrations was most consistent with a reduced clearance of *S*-ibuprofen, rather than enhanced inversion, because plasma concentrations of the *R*-enantiomer were unchanged. In view of the apparent predisposition of some individuals to toxicity, an appreciation for the enzymes responsible for ibuprofen oxidation would provide a rational basis for predicting the contribution of environmental and genetic factors to this increased risk. Interestingly, Leeman *et al.* [4] suggested that because sulfaphenazole was an effective inhibitor of *R*- and *S*-ibuprofen 2- and 3-hydroxylation CYP2C9 was solely responsible for catalyzing these biotransformations. However, it is difficult to reconcile a monoenzymatic catalysis of ibuprofen oxidation with the highly stereoselective changes in plasma concentrations observed in some individuals with renal impairment [3, 5].

Several lines of evidence suggest that CYP2C9 is indeed a quantitatively predominant enzyme in the catalysis of ibuprofen hydroxylation. First, cDNA-expressed CYP2C9 was clearly the most active individual CYP with K_m values (35–82 μM) close to those noted in microsomes (21–47 μM; Table 1). The enantioselectivity of the expressed CYP2C9 also paralleled that observed in microsomes; *S*:*R* ratios for intrinsic clearances were 1.3 and 1.8 for 2- and 3-hydroxylation, respectively (Fig. 4). Second, sulfaphenazole was a potent competitive inhibitor of ibuprofen

TABLE 4. Correlations between the activities of *R*- and *S*-ibuprofen hydroxylations in a bank of 14 liver microsomal samples

	R-2-Hydroxylation	*R*-3-Hydroxylation	*S*-2-Hydroxylation	*S*-3-Hydroxylation
R-2-Hydroxylation	1.00	0.75*	0.75*	0.67†
R-3-Hydroxylation		1.00	0.90*	0.98*
S-2-Hydroxylation			1.00	0.90*
S-3-Hydroxylation				1.00

* $P \leq 0.01$.
† $P \leq 0.05$.

hydroxylation with K_i estimates in the range of 0.08 to 0.16 μM (Fig. 3), and similar K_i values were noted for cDNA-expressed CYP2C9. These K_i values are comparable to the estimates obtained by Rettie *et al.* [22] for the competitive inhibition of S-warfarin 7-hydroxylation by sulfaphenazole in human liver microsomes (0.18 μM) and cDNA-expressed CYP2C9 (0.21 μM). Third, high correlations were observed between all ibuprofen hydroxylations and S-warfarin 7-hydroxylation, phenytoin 4′-hydroxylation, and tolbutamide methylhydroxylation, which are prototypical CYP2C9 biotransformations (Table 3; [22, 24, 29]).

The importance of CYP2C9 in the hydroxylation of ibuprofen is clear, but there are also indications that other CYPs may participate in these reactions. For example, the relationships between metabolite formation rate and substrate concentration are consistent with at least two enzymes for some microsomal preparations (Fig. 2). The correlations between the isomeric hydroxyibuprofen formation rates are also revealing. The rates of formation of S- and R-3-OHIbu are almost perfectly correlated ($r^2 = 0.96$), and, therefore, the lower correlations between 2- and 3-OHIbus are indicative of the involvement of multiple enzymes with independent regulation in these reactions.

Both tolbutamide and ibuprofen hydroxylations can be affected by CYP2C8 [29, 30]. Therefore, the biphasic nature of the Eadie–Hofstee plots for both tolbutamide and ibuprofen hydroxylations in some microsomal samples is consistent with a role for CYP2C8 in these biotransformations. Taxol 6α-hydroxylation has been identified as a selective substrate for CYP2C8 [17], and, therefore, the significant correlation between this activity and the hydroxylations of ibuprofen also suggests that CYP2C8 may catalyze these reactions. However, high correlations were noted between all the putative CYP2C9 selective activities and taxol 6α-hydroxylation. Correlations with other CYP activities reached statistical significance alone, but taxol 6α-hydroxylation was the only activity to remain significant ($P \leq 0.05$) in the presence of CYP2C9 activities when multiple linear regression analysis was employed. Direct evidence that CYP2C8 can hydroxylate R- and S-ibuprofen was provided by the cDNA-expressed enzyme, which displayed a clear regioselectivity in favor of R-2-hydroxylation and an intrinsic clearance value comparable to those of CYP2C9 (Fig. 4). Finally, the pattern of inhibition of ibuprofen hydroxylation in human liver microsomes is also consistent with a contribution of CYP2C8 to these biotransformations. Sulfaphenazole was found to be a selective inhibitor of cDNA-expressed CYP2C9 with a K_i range of 0.08 to 0.18 μM compared with a range of 0.36 to 0.55 μM for CYP2C8. This is consistent with previous reports showing inhibition of S-warfarin 7-hydroxylation by sulfaphenazole [22, 29] but little inhibition of CYP2C8-mediated reactions, such as taxol 6α-hydroxylation [29, 31]. In microsomes, sulfaphenazole inhibited the 3-hydroxylation with a K_i of 0.06 μM but the 2-hydroxylation with a K_i of 0.12 μM (Table 2). In contrast, arachidonic acid and retinoic acid, which are substrates for CYP2C8 [10, 23], did not exhibit regioselectivity in their inhibition of ibuprofen hydroxylations. Taken together these data suggest that the 3-hydroxylations of ibuprofen are almost exclusively CYP2C9 mediated, whereas the 2-hydroxylations depend on other CYPs, such as CYP2C8. This conclusion is consistent with the high correlation between the 3-hydroxylations but not between the 3- and 2-hydroxylations (Table 4).

In addition to the well-characterized members of the CYP2C subfamily, such as CYP2C8, 2C9, and 2C19, a number of variant proteins have been identified, and their prevalence and ethnoselectivity have been examined [29, 30, 32, 33]. For CYP2C9, several variants have been shown to display catalytic properties towards prototypical substrates that differ from those of the wild-type enzyme [29, 30, 33, 34]. For example S-warfarin 7-hydroxylation, tolbutamide methylhydroxylation, and phenytoin 4′-hydroxylation are less efficiently catalyzed by the Arg 144 → Cys 144 variant of CYP2C9 [29, 30, 33]. Similarly, the Ile 359 → Leu 359 variant exhibits reduced activity towards CYP2C9 substrates *in vitro* and *in vivo* where < 1% of Caucasians, Chinese–Taiwanese, and African–Americans are homozygous at this gene locus and correspond to the tolbutamide poor metabolizer phenotype [28, 32–35]. Approximately 20% of Caucasians are heterozygous for the mutation corresponding to the Cys 144 variant of CYP2C9, but it is absent from Chinese individuals, and, therefore, the potential for significant interethnic differences in CYP2C9 activity exists [32]. We have demonstrated that the Cys 144 variant of CYP2C9 displays intrinsic clearance values for ibuprofen hydroxylations markedly lower than those of the wild-type enzyme (Fig. 4) but exhibits comparable sulfaphenazole K_i values. The expression of this variant, therefore, would be expected to provide an additional source of interindividual and perhaps interethnic variability in ibuprofen disposition.

In summary, it is apparent that CYP2C9 is the major CYP mediating the 2- and 3-hydroxylations of R- and S-ibuprofen in Caucasian liver. Additional CYPs, particularly CYP2C8, may also play a role in these biotransformations, and thus the relative expression of CYP2C9, CYP2C8, and their variants would be expected to contribute to interindividual and interethnic variability in the clearance of ibuprofen enantiomers. For example, individuals expressing one or more catalytically less active variants of CYP2C9 may be characterized by an "average" clearance of R-ibuprofen but a reduced clearance of S-ibuprofen due the predominance of CYP2C8. CYP2C8 plays a central role in the enantioselective activation of arachidonic acid to EETs, which, in turn, have profound effects on ion transport and hemodynamic regulation. The potential inhibition of these CYP2C-catalyzed biotransformations by ibuprofen, and other NSAIDs, may constitute an additional, cyclooxygenase-independent mechanism of action.

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