Cytchrome P-450 2B6 Is Responsible for Interindividual Variability of Propofol Hydroxylation by Human Liver Microsomes

Michael H. Court,* Su X. Duan,† Leah M. Hesse,‡ Karthik Venkatakrishnan,§ David J. Greenblatt||

Background: Oxidation of propofol to 4-hydroxypropofol represents a significant pathway in the metabolism of this anesthetic agent in humans. The aim of this study was to identify the principal cytochrome P-450 (CYP) isoforms mediating this biotransformation.

Methods: Propofol hydroxylation activities and enzyme kinetics were determined using human liver microsomes and cDNA-expressed CYPs. CYP-specific marker activities and CYP2B6 protein content were also quantified in hepatic microsomes for correlational analyses. Finally, inhibitory antibodies were used to ascertain the relative contribution of CYPs to propofol hydroxylation by hepatic microsomes.

Results: Propofol hydroxylation by hepatic microsomes showed more than 19-fold variability and was most closely correlated to CYP2B6 protein content (r = 0.904), and the CYP2B6 marker activities, 5-methylenetetrahydrofolate dehydrogenase (r = 0.919) and bupropion hydroxylation (r = 0.854). High- and intermediate-activity livers demonstrated high-affinity enzyme kinetics (K_m < 8 μM), whereas low-activity livers displayed low-affinity kinetics (K_m > 80 μM). All of the CYPs evaluated were capable of hydroxylating propofol; however, CYP2B6 and CYP2C9 were most active. Kinetic analysis indicated that CYP2B6 is a high-affinity (K_m = 10 ± 2 μM; mean ± SE of the estimate), high-capacity enzyme, whereas CYP2C9 is a low-affinity (K_m = 41 ± 8 μM), high-capacity enzyme. Furthermore, immunoinhibition showed a greater contribution of CYP2B6 (56 ± 22% inhibition; mean ± SD) compared with CYP2C isoforms (16 ± 7% inhibition) to hepatic microsomal activity.

Conclusions: Cytchrome P-450 2B6, and to a lesser extent CYP2C9, contribute to the oxidative metabolism of propofol. However, CYP2B6 is the principal determinant of interindividual variability in the hydroxylation of this drug by human liver microsomes.

Propofol is commonly used for intravenous anesthesia and sedation of human patients. One of the major advantages of this drug over other injectable anesthetic agents is that relatively rapid and complete recovery occurs in most patients after repeated dosing or with relatively prolonged intravenous infusions.¹ This property is attributable, in part, to rapid and extensive biotransformation of propofol to multiple metabolites, primarily by the liver (fig. 1). The major route of metabolism appears to be glucuronidation of the parent compound at the sterically hindered C1-hydroxyl position.² To date, UDP-glucuronosyltransferase 1A9 is the only enzyme that has been shown to mediate this reaction.³ Propofol may also undergo ring hydroxylation by cytochrome P-450 (CYP) to form 4-hydroxypropofol, which is then glucuronidated at either the C1- or C4-hydroxyl positions or is sulphated at the C4-hydroxyl position by a sulphotransferase. Although the glucuronide and sulphate conjugates of propofol appear to be pharmacodynamically inactive, 4-hydroxypropofol is reported to have approximately one third of the hypnotic activity of propofol.⁴

A number of studies have attempted to quantitate the relative amounts of metabolites in the urine of humans administered propofol. Glucuronide and sulphate conjugates of 4-hydroxypropofol were found to average 60,⁵ 47,² 25,² and 24% of total urinary propofol metabolites in groups of four unidentified patients, six male white volunteers, eight Japanese patients (seven male, one female), and six male white patients, respectively. Importantly, there appears to be significant interindividual variation, with values for 4-hydroxypropofol conjugates ranging from as little as 7%² to as much as 72%⁵ of total urinary metabolites. Consequently, identification and characterization of the enzymes mediating the biotransformation of propofol may help to elucidate the molecular basis for these differences.

Using 11 different cDNA-expressed CYPs, it was recently shown that the hydroxylation of propofol can be mediated by multiple hepatic CYP isoenzymes.⁷ Based on the correlation of hepatic microsomal propofol hydroxylase activities to immunoquantified CYP2C9 levels and inhibition of this activity by a CYP2C9 antibody and sulphaphenazole, a purported selective CYP2C9 chemical inhibitor, it was concluded that CYP2C9 contributed by at least 50% to the oxidation of propofol. However, the correlation was not very strong (r = 0.78), inhibition by sulphaphenazole was relatively weak (< 39% inhibition), and immunoinhibition was only performed in livers containing a high amount of CYP2C9 protein. In addition, based on the presented data, the K_m value for propofol hydroxylation by CYP2C9 was estimated to be approximately 50 μM, almost three times higher than the mean K_m value for hepatic microsomes (18 μM). Although it is possible that no one CYP is primarily responsible for the oxidation of propofol, not all of the CYPs

* Assistant Professor, Department of Pharmacology and Experimental Therapeutics, Tufts University, and Department of Clinical Sciences, School of Veterinary Medicine, North Grafton, Massachusetts. † Research Technician, Department of Pharmacology and Experimental Therapeutics, Tufts University, and Department of Clinical Sciences, School of Veterinary Medicine, North Grafton, Massachusetts. ‡ Graduate Student, Department of Pharmacology and Experimental Therapeutics, Tufts University. § Research Career Award (grant No. K01-RR-00104) from the National Center for Research Resources, National Institutes of Health, Bethesda, Maryland.|| Submitted for publication December 27, 1999. Accepted for publication August 7, 2000. Supported by grants No. RR00104, MH54223, MH012357, and MH19924 from the National Institutes of Health, Bethesda, Maryland.|| Address reprint requests to Dr. Court: Department of Pharmacology and Experimental Therapeutics, Tufts University, 136 Harrison Avenue, Boston, Massachusetts 02111. Address electronic mail to: mcourt01@emerald.tufts.edu. Individual article reprints may be purchased through the Journal Web site, www.anesthesiology.org.
known to be expressed in human liver were evaluated. In particular, cDNA-expressed CYP2B6 was not studied, although involvement of this isofrom was excluded based on a lack of inhibition of hepatic microsomal activity by orphenadrine. However, orphenadrine is a relatively nonselective and weak inhibitor of CYP2B6, and the concentration used (50 μM) was low.8

In studies designed to investigate the molecular basis for dog breed differences in propofol metabolism, we determined that CYP2B11 is the principal hepatic propofol hydroxylase in dogs.9 Furthermore, propofol hydroxylation by rat liver microsomes is induced most strongly by pretreatment of rats with phenobarbital, also indicating potential involvement of a CYP2B isoform.10 Consequently, we postulated that the human hepatic CYP2B ortholog, CYP2B6, may also mediate propofol hydroxylation. In the present study, we used cDNA-expressed CYPs and a bank of liver tissue derived from 34 donors to ascertain and evaluate the role of CYP2B6 in the metabolism of propofol in humans.

Materials and Methods

Reagents

Pure propofol (2,6-diisopropylphenol) was provided by AstraZeneca Pharmaceuticals (Wilmington, DE), and a small quantity of pure 4-hydroxypropofol (2,6-diisopropyl-1,4-quinol) was a gift of Dr. J. Guitton (Université Claude Bernard, Lyon, France). Thymol, NADP⁺, isocitrate dehydrogenase, DL-isocitrate, dimethylsulfoxide, and 50 mM potassium phosphate buffer (pH 7.5) were purchased from Sigma (St. Louis, MO). Acetonitrile and methanol were obtained from Fisher Scientific (Fairlawn, NJ). N-mephenytoin, nirvanol and 4-hydroxymephénytoin were purchased from Gentest (Woburn, MA). The N-mephenytoin contained approximately 1% nirvanol and was therefore purified to apparent homogeneity (< 0.01% nirvanol) as previously described.11 Bupropion and hydroxybupropion were provided by GlaxoWellcome (Research Triangle Park, NC), and trazodone was obtained from Mead Johnson (Evansville, IN). R-flurbiprofen, 4-hydroxyflurbiprofen, and 2-fluoro-4-biphenyl acetic acid were gifts from Dr. Timothy Tracy (School of Pharmacy, West Virginia University, Morgantown, WV).

Recombinant CYP isoenzymes and vector control from a β-lymphoblastoid cell expression system were obtained from Gentest. Microsomal protein and total CYP concentrations were supplied by the manufacturer. Although the β-lymphoblastoid cell microsomes contain basal levels of both CYP oxidoreductase and cytochrome b5, CYPs 2A6, 2C9, 2D6, 2E1, and 3A4 were coexpressed with oxidoreductase, whereas CYPs 1A2, 2C19 and 2B6 were not available in this form. Monoclonal inhibitory antibody specific for CYP2B6 was also obtained from Gentest. In preliminary experiments in this laboratory and at Gentest, this antibody showed potent and specific inhibition of N-mephenytoin N-demethylation by human liver microsomes and cDNA-expressed CYP2B6 (> 90% inhibition at 1:10 w/w antibody to microsomal protein ratio). Minimal or no inhibition was observed for other activities specific for CYPs 1A2, 2A6, 2C8, 2C9, 2C18, 2C19, 2D6, 2E1, or 3A4. A second inhibitory monoclonal antibody specific for CYP2C isoforms was obtained from Pan Vera (Madison, WI). This antibody was found to potently inhibit (> 90% inhibition at 3:1 w/w antibody to microsomal protein ratio) all of the human CYP2C isoenzymes, including CYPs 2C8, 2C9, 2C18 and 2C19.

The liver tissues used in this study were obtained from either the National Disease Research Interchange (Philadelphia, PA) or the Liver Tissue Procurement and Distribution Service (Minneapolis, MN). Donors included 21 males and 13 females with ages ranging from 2 to 74 yr (median, 24 yr). Identified ethnicities included 24 whites, 7 blacks, and 3 Hispanic Americans. The majority of liver tissues were obtained from organ donor patients with a history of head trauma or cerebral anoxia. Liver tissues from 3 donors were obtained as biopsy specimens of apparently healthy tissue adjacent to either bladder or colon cancers. Light to moderate cigarette smoking and alcohol consumption histories were reported for 10 and 7 donors, respectively. Microsomes were prepared from liver tissues as previously described.12 The final microsomal pellet was resuspended in phosphate buffer containing 20% glycerol and stored at −80°C. An aliquot of this suspension was used to determine protein concentration by the bicinchoninic acid assay technique (Pierce, Rockford, IL).

In Vitro Biotransformation Assays

Typical in vitro incubations consisted of hepatic microsomes or cDNA-expressed CYPs, drug substrate, and an nicotinamide adenine dinucleotide phosphate...
Enzyme Kinetic Analysis

Propofol hydroxylation, S-mephenytoin N-demethylation, and S-mephenytoin 4-hydroxylation activities were determined as described in detail previously. Propofol concentrations were between 1 and 100 μM, with a microsomal protein concentration of 0.4 mg/ml and incubation time of 10 min. Propofol was solubilized in dimethylsulfoxide (0.2% final concentration). In a preliminary study there was no difference in propofol hydroxylation activity in incubates containing 0.4% compared with 0.2% dimethylsulfoxide. S-mephenytoin concentration was 250 μM, microsomal protein concentration was 0.5 mg/ml, and incubation time was 120 min. R-flurbiprofen hydroxylation activity was assayed as described previously except that the organic extraction step was omitted. Substrate concentration was 250 μM, microsomal protein concentration was 0.4 mg/ml, and incubation time was 20 min. Finally, bupropion hydroxylation activities were determined using an adaptation of the method for measuring bupropion and its metabolites in plasma described by Cooper et al. Incubations were performed for 20 min with a substrate concentration of 250 μM and a microsomal protein concentration of 0.25 mg/ml. Trazodone was used as the internal standard. The mobile phase was 50 mM potassium phosphate buffer (pH = 3.0) with acetonitrile (79:21 vol/vol), and the column effluent was monitored by ultraviolet absorbance at a wavelength of 214 nm.

Immunoinhibition

An immunoinhibition study of propofol hydroxylation activity in hepatic microsomes was performed using the monoclonal anti-CYP2B6 and anti-CYP2C antibodies. Microsomes (10–50 μg protein) were preincubated with antibody (1:1 or 3.5:1 wt/wt antibody to microsomal protein ratio for anti-CYP2B6 or anti-CYP2C antibodies, respectively) for 30 min at room temperature in a volume of 50 μl. The antibody to microsome protein ratio used had been determined in previous experiments to result in a maximal degree of inhibition. Control reactions contained an equal volume of the antibody diluent (Tris buffer). The reaction was then started as usual by adding propofol (final concentration 5 μM) and the NADPH cofactor solution, and processed as described previously for analysis of metabolite formation by HPLC.

CYP2B6 Immunoblot

Microsomal CYP2B6 content was quantitated by adapting a previously described immunoblotting method. Briefly, hepatic microsomal protein (10–50 μg) and lymphoblast-expressed CYP2B6 (0.05–2.5 pmoles) were separated by sodium dodecylsulfate–polyacrylamide gel electrophoresis using precast 7.5% gels (Biorad, Hercules, CA). Proteins were then electrophoretically transferred to polyvinyl difluoride membrane (Immobilon-P, Millipore, Bedford, MA). These blots were blocked with 0.5% powdered nonfat milk in TBS-Tween (0.15 M NaCl, 0.04 M Tris, pH 7.7, and 0.06% Tween 20) and then incubated in TBS-Tween/0.1% bovine serum albumin containing a 1:500 dilution of a polyclonal antipeptide CYP2B6 antibody (Gentest). After washing, the blots were incubated in a 1:500 dilution of horse radish peroxidase conjugated secondary antibody (Gentest), washed, and then chemiluminesence detection was performed (Super Signal Substrate; Pierce, Rockford, IL) with exposure to radiographic film (Biomax MR; Kodak, Rochester, NY). The film was then scanned, and the area

(NADPH) regeneration system in a final volume of 250 μl of phosphate buffer as described in detail previously with modifications indicated below.12 Incubation time (at 37°C) and microsomal protein content were minimized to ensure linearity of product formation with respect to these variables. Metabolite formation was quantitated by high-performance liquid chromatography (HPLC). HPLC apparatus were obtained from Waters (Milford, MA) and consisted of a dual-head pump with autoinjector (Models 515 and 717) serially connected to a 300- × 3.9-mm ID reverse-phase C-18 Bondapack column with either an ultraviolet absorbance (Model 486) or fluorescence detector (Model 474) depending on the assay method used.

Propofol hydroxylation, S-mephenytoin N-demethylation, and S-mephenytoin 4-hydroxylation activities were determined as described in detail previously. Propofol concentrations were between 1 and 100 μM, with a microsomal protein concentration of 0.4 mg/ml and incubation time of 10 min. Propofol was solubilized in dimethylsulfoxide (0.2% final concentration). In a preliminary study there was no difference in propofol hydroxylation activity in incubates containing 0.4% compared with 0.2% dimethylsulfoxide. S-mephenytoin concentration was 250 μM, microsomal protein concentration was 0.5 mg/ml, and incubation time was 120 min. R-flurbiprofen hydroxylation activity was assayed as described previously except that the organic extraction step was omitted. Substrate concentration was 250 μM, microsomal protein concentration was 0.4 mg/ml, and incubation time was 20 min. Finally, bupropion hydroxylation activities were determined using an adaptation of the method for measuring bupropion and its metabolites in plasma described by Cooper et al. Incubations were performed for 20 min with a substrate concentration of 250 μM and a microsomal protein concentration of 0.25 mg/ml. Trazodone was used as the internal standard. The mobile phase was 50 mM potassium phosphate buffer (pH = 3.0) with acetonitrile (79:21 vol/vol), and the column effluent was monitored by ultraviolet absorbance at a wavelength of 214 nm.

Enzyme Kinetic Analysis

Propofol hydroxylation activities were determined using liver microsomes and cDNA-expressed CYPs with propofol concentrations varying between 1 and 100 μM. Data were fit to the appropriate kinetic models by non-linear regression analysis (SigmaPlot, SPSS, Chicago, IL). In most instances, data were adequately described by the simple Michaelis-Menten relation:

\[ V = \frac{V_{\text{max}} \times S}{K_m + S} \]  

where \( V_{\text{max}} \) is the maximal reaction velocity, \( K_m \) is the Michaelis-Menten constant, \( S \) is propofol concentration, and \( V \) is the rate of formation of 4-hydroxypropofol. For some sets of data, Eadie-Hofstee plots were concave and were analyzed using a two-enzyme model:

\[ V = \frac{V_{\text{max1}} \times S}{K_{m1} + S} + \frac{V_{\text{max2}} \times S}{K_{m2} + S} \]  

where subscripts 1 and 2 represent \( V_{\text{max}} \) and \( K_m \) values for apparent high- and low-affinity activities. For other sets of data, Eadie-Hofstee plots were convex, \( V \) versus \( S \) plots were sigmoidal rather than hyperbolic, and these were analyzed using the Hill equation:

\[ V = \frac{V_{\text{max}} \times S^n}{K_m^n + S^n} \]  

where the exponent (\( n \)) indicates the degree of sigmoidicity.

Immunoinhibition

An immunoinhibition study of propofol hydroxylation activity in hepatic microsomes was performed using the monoclonal anti-CYP2B6 and anti-CYP2C antibodies. Microsomes (10–50 μg protein) were preincubated with antibody (1:1 or 3.5:1 wt/wt antibody to microsomal protein ratio for anti-CYP2B6 or anti-CYP2C antibodies, respectively) for 30 min at room temperature in a volume of 50 μl. The antibody to microsome protein ratio used had been determined in previous experiments to result in a maximal degree of inhibition. Control reactions contained an equal volume of the antibody diluent (Tris buffer). The reaction was then started as usual by adding propofol (final concentration 5 μM) and the NADPH cofactor solution, and processed as described previously for analysis of metabolite formation by HPLC.

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and density of identified bands were quantitated (NIH Image 1.62 software, National Institutes of Health, Bethesda, MD). Hepatic microsomal CYP2B6 content was then calculated from the standard curve data, which were fit by nonlinear least squares regression to the equation \( y = m \times \ln(x) + b \), where \( x \) is CYP2B6 content and \( y \) is the product of band area and density.

**Statistical Analysis**

Statistical analyses were performed using the SigmaStat software package (Jandel Scientific, SPSS, Chicago, IL). The influence of ethnicity, gender, and age on propofol hydroxylation activities was evaluated by analysis of variance. Because initial analysis showed that data sets failed tests of normality of distribution (Kolmogorov-Smirnov test), the model (equations 1, 2, or 3) that best described the set of data was substantiated by comparison of the Pearson product-moment correlation coefficients (\( r \)). Finally, inhibition of propofol hydroxylation by anti-CYP2B6 and anti-CYP2C antibodies was compared by a paired one-tailed \( t \) test.

**Results**

Propofol hydroxylation activities were measured in microsomes from 34 different livers at 5- and 50-\( \mu \)M substrate concentrations. The lower concentration used was intended to approximate plasma propofol concentrations reported to be clinically relevant (total intravenous anesthesia: 10–20 \( \mu \)M\(^{19,20}\), sedative infusions: \(< 10 \mu \)M\(^{21}\)). As shown in figure 2, at 5 \( \mu \)M propofol there was 19-fold variation between the highest and lowest activity values (median, 0.91 nmoles \( \cdot \) min\(^{-1} \) \cdot mg\(^{-1} \); range, 0.25–4.8 nmoles \( \cdot \) min\(^{-1} \) \cdot mg\(^{-1} \)), whereas at 50 \( \mu \)M propofol there was an eightfold variation (median, 3.3 nmoles \( \cdot \) min\(^{-1} \) \cdot mg\(^{-1} \); range, 1.4–12 nmoles \( \cdot \) min\(^{-1} \) \cdot mg\(^{-1} \)). In both instances, the data were not normally distributed (Kolmogorov-Smirnov normality test; \( P < 0.05 \)), with significant skewing of data toward lower-activity values. No significant influence of gender, ethnicity, or age on propofol hydroxylation activities measured at 5- and 50-\( \mu \)M substrate concentration could be discerned. However, the small size of the minority ethnic groups and the widely dispersed age distribution limited the power of the statistical evaluation.

Enzyme kinetic analyses were then performed using microsomes from six different livers chosen to represent a range of propofol hydroxylation activities, including those with relatively high (HL33 and HL34), intermediate (HL25 and HL28), and low activities (HL6 and HL9). As illustrated in figure 3, kinetic data were adequately described by the single enzyme kinetic model (equation 1) in all but one liver (HL25), which was best described by the two-enzyme model (equation 3). Values for the fitted kinetic parameters are given in table 1. In the high activity livers, values for \( K_m \) were less than 8 \( \mu \)M, whereas in the low-activity livers, \( K_m \) values were more than 10 times higher (> 80 \( \mu \)M). In liver HL25, both high- and low-affinity components could be distinguished.

To identify CYP isoforms contributing to the observed hepatic microsomal propofol hydroxylation, cDNA-expressed CYPs were screened for this activity (table 2). At 5 \( \mu \)M substrate concentration, activity could be detected with all CYPs evaluated except for CYP2D6. CYP2B6

**Fig. 2.** Comparative rates of 4-hydroxypropofol formation in vitro by human liver microsomes derived from 34 different donors incubated in the presence of propofol and an NADPH-regenerating system. Representative livers that were selected for further study are indicated with an asterisk. Insets show the frequency distribution of the activities in this set of livers measured at 5 (upper) and 50 (lower) \( \mu \)M propofol.
from CYP2C9 were sigmoidal and best described by the Hill enzyme equation (equation 2), with an apparent $K_m$ of $41 \pm 8 \mu M$, a $V_{\text{max}}$ of $54 \pm 4 \text{nmoles} \cdot \text{min}^{-1} \cdot \text{mg}^{-1} \text{protein}$, and an exponent ($n$) value of 1.5 $\pm$ 0.2.

Next, marker activities proposed to be specific for CYP2B6 ($S$-mephenytoin $N$-demethylation$^{22}$ and bupropion hydroxylation$^{23}$) and CYP2C9 ($R$-flurbiprofen hydroxylation$^{14}$) were determined using the entire set of liver microsomes. Rates of $S$-mephenytoin 4-hydroxylation, a marker activity for CYP2C19, were also measured coincidentally with $S$-mephenytoin $N$-demethylation measurements. Correlational analyses were then performed (table 3). As shown in figure 5, at a 5-$\mu M$ substrate concentration, propofol hydroxylation was highly correlated with $S$-mephenytoin $N$-demethylation ($r = 0.925$), bupropion hydroxylation ($r = 0.854$), and immunquantified CYP2B6 content ($r = 0.904$) but not with $R$-flurbiprofen hydroxylation ($r = 0.237$). Removal of the two highest propofol hydroxylase activity livers (HL33 and 34) from the analysis reduced $r$ values for all comparisons. However, correlation coefficients were still higher for CYP2B6 marker activities ($r = 0.767$ and 0.762 for $S$-mephenytoin $N$-demethylation and bupropiion hydroxylation, respectively).

Clearly, the 5-$\mu M$ concentration of propofol was sufficient to activate CYP2C9 and CYP2B6 for hydroxylation of propofol. Hence, with a single concentration of propofol, the activities of all indicated CYPs can be determined.

**Table 1. Enzyme Kinetic Values for Propofol 4-Hydroxylation by Liver Microsomes**

<table>
<thead>
<tr>
<th>Liver</th>
<th>$K_m_1$ ($\mu M$)</th>
<th>$V_{\text{max}}_1$ (nmoles $\cdot$ min$^{-1}$ $\cdot$ mg$^{-1}$ protein)</th>
<th>$K_m_2$ ($\mu M$)</th>
<th>$V_{\text{max}}_2$ (nmoles $\cdot$ min$^{-1}$ $\cdot$ mg$^{-1}$ protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HL6</td>
<td>8.6 $\pm$ 12</td>
<td>5.6 $\pm$ 0.5</td>
<td>140 $\pm$ 70</td>
<td>10 $\pm$ 2</td>
</tr>
<tr>
<td>HL9</td>
<td>9.4 $\pm$ 7</td>
<td>4.8 $\pm$ 0.2</td>
<td>10 $\pm$ 0.3</td>
<td></td>
</tr>
<tr>
<td>HL25*</td>
<td>3.7 $\pm$ 2.6</td>
<td>1.3 $\pm$ 0.6</td>
<td>8.4 $\pm$ 0.3</td>
<td></td>
</tr>
<tr>
<td>HL28</td>
<td>8.7 $\pm$ 1.0</td>
<td>10 $\pm$ 0.3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HL33</td>
<td>7.8 $\pm$ 1.1</td>
<td>8.4 $\pm$ 0.3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HL34</td>
<td>7.4 $\pm$ 0.9</td>
<td>13 $\pm$ 0.4</td>
<td></td>
<td></td>
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</tbody>
</table>

Shown are the fitted values followed by the standard errors of the estimates. * Best described by the two-enzyme kinetic model (equation 2), in which subscripts 1 and 2 indicate kinetic values for high- and low-affinity enzymes, respectively.

$K_m$ = Michaelis-Menten constant; $V_{\text{max}}$ = maximal reaction velocity.

**Table 2. Propofol 4-Hydroxylation Activities Measured at 5 and 50 $\mu M$ Substrate Concentration Using Microsomes from $\beta$-Lymphoblastoid Cells Expressing Individual CYP Isoenzymes**

<table>
<thead>
<tr>
<th>Expressed CYP Isoenzyme</th>
<th>5 $\mu M$ Propofol</th>
<th>50 $\mu M$ Propofol</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vector control</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>1A2</td>
<td>0.92</td>
<td>4.70</td>
</tr>
<tr>
<td>2A6</td>
<td>0.41</td>
<td>2.10</td>
</tr>
<tr>
<td>2B6</td>
<td>8.60</td>
<td>17.32</td>
</tr>
<tr>
<td>2C9</td>
<td>1.66</td>
<td>18.86</td>
</tr>
<tr>
<td>2C19</td>
<td>0.87</td>
<td>2.09</td>
</tr>
<tr>
<td>2D6</td>
<td>ND</td>
<td>0.22</td>
</tr>
<tr>
<td>2E1</td>
<td>0.04</td>
<td>0.34</td>
</tr>
<tr>
<td>3A4</td>
<td>0.06</td>
<td>1.31</td>
</tr>
</tbody>
</table>

Data represent the average of duplicate determinations.

CYP = cytochrome P450; ND = not detectable.
Hofstee transformation of these data. Vnmoles with a Hill exponent (n) of 1.5 for CYP2C9. (Bottom) and were 41
6 mg concentration at that velocity, nmoles
1 nmole
2
1 nmole
nmole

Fig. 4. (Top) The effect of substrate concentration on propofol hydroxylase activity in microsomes from β-lymphoblastoid cells expressing either CYP2B6 or CYP2C9. Activities are expressed as the rate of 4-hydroxypropofol formation normalized to incubation time and microsomal protein content. The solid line connects best-fit estimates of these data determined by nonlinear least-squares curve fit to either equation 1 (CYP2B6) or equation 3 (CYP2C9). Estimates for Km and Vmax were 10 ± 2
m and 21 ± 1 nmoles·min⁻¹·nmole⁻¹ CYP for CYP2B6, and were 41 ± 8
m and 34 ± 4 nmoles·min⁻¹·nmole⁻¹ CYP with a Hill exponent (n) of 1.5 for CYP2C9. (Bottom) Eadie-Hofstee transformation of these data. V = reaction velocity, nmoles·mg⁻¹·min⁻¹. V/[S] = reaction velocity divided by propofol concentration at that velocity, nmoles·mg⁻¹·min⁻¹·μM⁻¹.

pion hydroxylation, respectively) compared with CYP2C9 mediated flurbiprofen hydroxylation (r = 0.144). Multiple linear regression analysis was also performed using bupropion hydroxylation and flurbiprofen hydroxylation activities as the independent variables, which resulted in a slight improvement in correlation coefficient (r = 0.868). At the higher propofol concentration, correlation values were slightly lower for both CYP2B6 marker activities and slightly higher for the CYP2C9 marker activity (table 3).

Finally, immunoinhibition was used to determine the relative contribution of CYP2B6 and CYP2C isoforms to propofol hydroxylation at the predicted clinically relevant substrate concentration of 5 μM. The liver microsomes used in this study were the same as those previously used to determine the kinetics of propofol hydroxylation. Figure 6 shows the absolute and relative (expressed as a percent of total) propofol hydroxylation activities that were inhibited by either the anti-CYP2B6 or the anti-CYP2C antibodies in each of the six livers. Mean (± SD) percent inhibition was almost threefold higher (P = 0.003) for the CYP2B6 antibody (56 ± 22% compared with the CYP2C antibody (16 ± 7%). If it is assumed (as was indicated by preliminary studies) that these antibodies are capable of completely inhibiting isoform-dependent activity, in the lower activity livers (HL6 and HL9) both CYP2B6 and CYP2C isoforms account for less than 50% of the total activity. On the other hand, in the remaining intermediate- and high-activity livers, the combined contribution of these isoforms reached as high as 96% of total activity. However, when the relative contributions of CYP2B6 and CYP2C isoforms were compared in these livers, CYP2B6 predominated, accounting for 55–75% of total propofol hydroxylation activity, in contrast to CYP2C isoforms, which accounted for less than 25% of this activity.

Discussion

Although CYP2B6, and to a lesser extent CYP2C9, contribute to propofol hydroxylation by human liver microsomes, CYP2B6 is clearly the principal determinant of interindividual differences at clinically relevant substrate concentrations (< 20 μM) based on the following evidence. In the 34 different livers evaluated, there was more than 19-fold variability in propofol hydroxylation. Representative high- and intermediate-activity livers demonstrated high-affinity enzyme kinetics (Km < 8 μM), whereas low-activity livers displayed low-affinity kinetics (Km > 80 μM). Consequently, the observed activity differences between livers most likely result from the variable contribution of a high-affinity high-capacity propofol hydroxylase. Although all cDNA-expressed CYPs were capable of hydroxyrating propofol, CYP2B6 and CYP2C9 were most active. Furthermore, kinetic analysis of these CYPs indicated that CYP2B6 is a high-affinity (Km = 10 μM), high-capacity enzyme, whereas CYP2C9 is a low-affinity (Km = 41 μM), high-capacity enzyme. The contribution of CYP2B6 to interindividual variability in propofol hydroxylation by hepatic microsomes was further substantiated by showing a strong correlation to CYP2B6 marker activities (r > 0.85) and CYP2B6 protein content (r = 0.904) as well as substantial inhibition by a CYP2B6-specific antibody, especially in the intermediate- and high-activity livers. In the low-activity livers, no single CYP isoform could be discerned, suggesting that multiple CYPs in addition to CYP2B6 and CYP2C9 contribute to the observed activity.
Our results are somewhat different from those reported by Guitton et al.\textsuperscript{7} Although median propofol hydroxylation activities were similar between the two studies, we found a much higher interindividual variability (19-fold vs. twofold). This difference may relate to the number of livers studied (12 in the study by Guitton et al. vs. 34 in the present study), affecting the likelihood of finding slow and fast metabolizers. Other possibilities include differences in liver donor demographics (all whites in the study by Guitton et al. vs. multiple ethnicities in the present study), liver quality, and assay methodologies. Guitton et al. also found a reasonable correlation (r = 0.78) between microsomal propofol hydroxylation and CYP2C9 protein content, and inhibition (47% decrease in activity) by a CYP2C9 antibody. However, immunoinhibition was only performed in three livers with a high CYP2C9 content, and inhibition by the CYP2C9 selective compound, sulphaphenazole, was modest (1-39% decrease in activity). In agreement with the study by Guitton et al., we found that CYP2C9 is clearly a proficient propofol hydroxylase, especially at higher propofol concentrations. In contrast, we only found a weak correlation to the CYP2C9 marker activity (flurbipron hydroxylation; r = 0.237) and modest im-

Fig. 5. Comparison of propofol hydroxylation activities to activities mediated by CYP2B6 (S-mephenytoin N-demethylation and bupropion hydroxylation) and CYP2C9 (R-flurbiprofen hydroxylation), as well as immunoquantified CYP2B6 protein content measured in the same set of human liver microsomes (n = 34 for comparisons between activities; n = 12 for comparison to immunoquantified CYP2B6). Also shown is the Pearson product-moment correlation value (r) for each comparison.

<table>
<thead>
<tr>
<th>S-mephenytoin N-demethylation</th>
<th>Bupropion Hydroxylation</th>
<th>R-flurbiprofen Hydroxylation</th>
<th>S-mephenytoin 4-Hydroxylation</th>
<th>CYP2B6 Protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>Propofol 4-hydroxylation</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5 μM</td>
<td>0.925</td>
<td>0.854</td>
<td>0.237</td>
<td>0.061</td>
</tr>
<tr>
<td>50 μM</td>
<td>0.779</td>
<td>0.661</td>
<td>0.357</td>
<td>0.030</td>
</tr>
<tr>
<td>S-mephenytoin N-demethylation</td>
<td></td>
<td>0.938</td>
<td>0.143</td>
<td>0.029</td>
</tr>
<tr>
<td>Bupropion hydroxylation</td>
<td></td>
<td>0.059</td>
<td>0.003</td>
<td>0.989</td>
</tr>
<tr>
<td>R-flurbiprofen hydroxylation</td>
<td></td>
<td></td>
<td>0.065</td>
<td>-0.237</td>
</tr>
<tr>
<td>S-mephenytoin 4-hydroxylation</td>
<td></td>
<td></td>
<td></td>
<td>-0.431</td>
</tr>
</tbody>
</table>

* Values represent the Pearson product-moment correlation coefficient (r) for the indicated comparisons using the same set of liver microsomes (n = 34 for comparisons between activities; n = 12 for comparisons to immunoquantitated CYP2B6).

CYP = cytochrome P450.

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munoinhibition (< 25%), suggesting a relatively minor contribution of CYP2C9 to propofol oxidation in the majority of the livers, at least at clinically relevant concentrations. On the other hand, we also found that correlation of propofol hydroxylation with S-mephenytoin N-demethylation (r = 0.919) was slightly stronger than with bupropion hydroxylation (r = 0.854). S-mephenytoin N-demethylation, although initially proposed as a pure CYP2B6 marker activity, has recently been shown to be mediated, in part, by CYP2C9,11 whereas bupropion hydroxylation appears to be a more specific CYP2B6 reaction.23 By use of multiple linear regression, we also showed a slight improvement in correlation to propofol hydroxylation activities when both CYP2B6 and CYP2C9 marker activities were incorporated into the regression equation (r = 0.85 for bupropion hydroxylation alone; r = 0.87 for bupropion hydroxylation with flurbiprofen hydroxylation). Consequently, CYP2C9 appears to provide a somewhat minor but discernable contribution to propofol hydroxylation in the majority of livers at clinically relevant concentrations. Its role is probably greater at higher substrate concentrations and in livers containing relatively high amounts of this enzyme.

The role of CYP2B6 in human xenobiotic metabolism appears to be underappreciated, probably because of a lack of availability (until relatively recently) of isoform-specific inhibitors and substrate probes.24 Compounding this has been the failure by many investigators to routinely include this isoform when screening recombinant CYPs for evidence of a particular activity. Admittedly, the relative abundance of CYP2B6 in the majority of livers may be low compared with other hepatic CYP isoforms, with estimates ranging from less than 1% to as much as 6% of total hepatic CYP.17 This is similar to estimates previously reported for CYP2E1 (6.6%), CYP2A6 (4%), and CYP2D6 (1.5%), but less than for CYP1A2 (12.7%), CYP2C (18.2%), and CYP3A (28.8%).25 However, it should be noted that in addition to relative isoform abundance, the probability of a particular CYP isoform contributing to a biotransformation reaction in vivo is also dependent on substrate affinity and concentration.

Although the average hepatic content of CYP2B6 is relatively low, there is evidence for considerable interindividual variability in CYP2B6 levels, which is likely to have important clinical consequences. Estimates based on either marker activities or immunodetectable protein range from 20-fold variation (consistent with data from this study) to as much as 100-fold variation.17,26–28 The cause of this variability in CYP2B6 expression is currently unknown but is likely to result from extrinsic (through the diet or by drug administration) or intrinsic (genetic) influences. CYP2B enzymes in most animals are distinguished by a susceptibility to induction by a variety of compounds, including phenobarbital, dexamethasone, peroxisome proliferators, and related compounds.24,29 Although relatively little is known with regard to induction of human CYP2B6, the gene promoter of this enzyme has recently been shown to be activated by phenobarbital-type inducers through the nuclear CAR receptor, much like the rat and mouse CYP2B isoforms.30 Unfortunately, none of the donors of the liver tissue used in this study had a history of exposure to any of these type of drugs, which might have accounted for the high activity observed in two of the livers (HL33 and 34), although these data could be incomplete. To date, no functionally relevant genetic polymorphisms of CYP2B6 have been identified. However, aberrant splicing of the primary CYP2B6 gene transcript has been described, which results in generation of a high proportion of nonfunctional enzyme.31,32 Although both nor-

Fig. 6. Inhibition of propofol hydroxylation activity in human liver microsomes by monoclonal antibodies specific for either CYP2B6 or human CYP2C isoforms. Propofol hydroxylation activities were measured in the presence of optimally inhibitory concentrations of each antibody at a substrate concentration of 5 μM. These values were then subtracted from control activities, measured in the presence of antibody diluent, to derive the absolute (top) and relative (bottom) amounts of activity inhibitable by either CYP2B6 or CYP2C antibodies.
mal and abnormal transcripts appear to occur in the tissues of all individuals, observed qualitative differences in the proportion of normal to abnormal transcripts could have resulted from the presence of a genetic polymorphism. However, the skewed distribution of the propofol hydroxylation values that we found in this study (and has been reported for CYP2B6 in other studies) is not consistent with a simple genetic polymorphism, which more commonly results in either a bi-modal or trimodal distribution of phenotype data. Consequently, CYP2B6 expression is more likely to be influenced by complex genetic or environmental interactions.

A significant amount of propofol appears to be cleared by extrahepatic tissues, especially in the lung, where first-pass uptake is as high as 60% in experimental models. Expression of CYP2B6 has been demonstrated in lung, kidney, intestine, and brain, whereas CYP2C9 has been detected in kidney and intestine but not in lung or brain. Consequently, it is possible that CYP2B6 may contribute to the extrahepatic clearance of propofol, especially in the lung, although confirmation of this will require further experimentation.

Propofol hydroxylation by recombinant CYP2C9, but not CYP2B6, showed sigmoidal enzyme kinetics. However, microsomal kinetics did not show sigmoidicity probably because the contribution of CYP2C9 was overwhelmed by other CYP isoforms. Although atypical kinetics are most commonly associated with oxidations by the CYP3A enzymes, this property is not unique to this CYP subfamily. For example, sigmoidal kinetics have recently been described for naphthalene metabolism by expressed CYPs 2B6, 2C8, 2C9, and 3A5, as well as dapsone metabolism by CYP2C9. Interestingly, dapsone was also shown to activate flurbiprofen and naproxen metabolism by CYP2C9, much like the activation of CYP3A4 metabolism by 7,8-benzoflavone. Such phenomena were explained by invoking a two-site model in which the enzyme can simultaneously bind two substrate molecules either within the same active site, or at two distinct binding sites, one of which is the active site and the second is a modulatory site. Because propofol is a relatively small molecule, it is possible that more than one molecule can bind to a sufficiently large enzyme active site. Excessive nonspecific binding of substrate to the microsomal matrix can also result in sigmoidal kinetics, but is unlikely in this case because CYP2B6 showed typical hyperbolic kinetics.

In conclusion, these studies have substantiated that CYP2B6, and to a lesser extent CYP2C9, contribute to the biotransformation of propofol. Furthermore, CYP2B6 appears to be the principal determinant of interindividual variability in the oxidation of this drug by human liver microsomes. Consequently, genetic and environmental factors affecting CYP2B6-mediated catalysis are predicted to have an influence on propofol biotransformation in humans.

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