Cytochrome P450 2B6 Catalyzes the Formation of Pharmacologically Active Sibutramine (N-{1-[1-(4-chlorophenyl)cyclobutyl]-3-methylbutyl}-N,N-dimethylamine) Metabolites in Human Liver Microsomes

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ABSTRACT:
We identified cytochrome P450 (P450) isozymes that are involved in the formation of two active sibutramine (N-{1-[1-(4-chlorophenyl)cyclobutyl]-3-methylbutyl}-N,N-dimethylamine) metabolites, M1 (N-{1-[1-(4-chlorophenyl)cyclobutyl]-3-methylbutyl}-N-methylamine) and M2 (1-[1-(4-chlorophenyl)cyclobutyl]-3-methylbutylamine), in humans using a combination chemical inhibition, correlation analyses in human liver microsomes (HLMs), and activity assays using recombinant P450s. Mechanism-based CYP2B6 inhibitors (i.e., clopidogrel, ticlopidine, and triethylenethiophoramide) significantly inhibited the formation of M1 from sibutramine and M2 from M1, respectively; in contrast, no effect was observed when using potent inhibitors of eight P450 isozymes (CYP1A2, CYP2A6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, CYP2E1, and CYP3A). In addition, the formations of M1 from sibutramine (r = 0.694, p = 0.0029) and M2 from M1 (r = 0.834, p < 0.0001) were strongly correlated with CYP2B6-catalyzed bupropion hydroxylation in 16 different HLM panels. Furthermore, recombinant CYP2B6 catalyzed M1 and/or M2 formation at the highest rate among 10 P450s. Although recombinant CYP2C19, 3A4, and 3A5 also catalyzed, to a less extent, M1 formation at high substrate concentrations (>5 μM), those contributions might be minor considering usual concentrations of sibutramine and M1 in the clinical setting. The kinetics of M1 and/or M2 formation from sibutramine in HLMs were fitted by a two-enzyme model, and the mean apparent K_m value (4.79 μM) for high-affinity component was similar to that observed in recombinant CYP2B6 (8.02 μM). In conclusion, CYP2B6 is the primary catalyst for the formation of sibutramine two active metabolites, which may suggest that pharmacogenetics and drug interactions of sibutramine in relation to CYP2B6 activity should be considered in the pharmacotherapy of sibutramine.

Sibutramine (Fig. 1) hydrochloride monohydrate is one of the few established treatment for obesity (Arterburn et al., 2004; Ioannides-Demos et al., 2006). As a central serotonergic and noradrenergic reuptake inhibitor, sibutramine activates a combination of serotoninergic and noradrenergic mechanisms to increase satiety and energy expenditure and decrease body weight (Heal et al., 1998; Luque and Rey, 1999).

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ABBREVIATIONS: Sibutramine, N-{1-[1-(4-chlorophenyl)cyclobutyl]-3-methylbutyl}-N,N-dimethylamine; M1 and M2, active metabolites of sibutramine; M1, N-{1-[1-(4-chlorophenyl)cyclobutyl]-3-methylbutyl}-N-methylamine; M2, 1-[1-(4-chlorophenyl)cyclobutyl]-3-methylbutylamine; P450, cytochrome P450; HLM, human liver microsome; thio-TEPA, triethylenethiophoramide; LC/MS/MS, liquid chromatography/tandem mass spectrometry; K_m, theoretical maximum inactivation rate constant; k_inact, initial slope; C_L, intrinsic clearance; C_max, peak plasma concentration.
hypertension (Luscombe et al., 1989; Stock, 1997). For this reason, M1 and M2 are thought to be safer than sibutramine itself (Glick et al., 2000).

To date, very little information is available regarding sibutramine metabolism. The manufacturer’s information (package insert for Meridia, a brand name of sibutramine; Knoll Pharmaceutical Co., 1997) implicates CYP3A4 in the formation of M1 and M2, but no published data are available. Therefore, our aim is to determine which P450 isoforms are involved in the formation of the pharmacologically active sibutramine metabolites, M1 and M2, in human liver microsomes (HLMs). We used selective chemical inhibitors to identify candidate enzymes and then examined the correlation between M1/M2 formation and P450 activity using well established marker activities in HLMs. Finally, we analyzed the catalytic activity of recombinant P450s in vitro. This information is important in understanding interindividual variability in the pharmacokinetics of sibutramine and its metabolites and in predicting drug-drug interactions. We also examined the utility of sibutramine metabolism as a marker of CYP2B6 activity.

Materials and Methods

Chemicals. Sibutramine, M1, and M2 were kindly donated by Yuhan Research Center of Yuhan Corporation (Kunpo, South Korea). Clopidogrel, coumarin, diethyldithiocarbamate, furafylline, ketoconazole, propranolol, quercetin, quinidine, sulfaphenazole, testosterone, ticlopidine hydrochloride, triethylenethiophosphoramide (thio-TEPA), and MgCl2 were purchased from Sigma-Aldrich Corporation (St. Louis, MO). MgCl2 was purchased from Sigma-Aldrich Corporation (St. Louis, MO). Clopidogrel, and MgCl2 were purchased from Sigma-Aldrich Corporation (St. Louis, MO). Research Center of Yuhan Corporation (Kunpo, South Korea). Clopidogrel, and MgCl2 were purchased from Sigma-Aldrich Corporation (St. Louis, MO).

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Kinetic Analysis in HLMs. The optimal conditions for microsomal incubation were selected based on the linear range of M1 and M2 formation from sibutramine and M2 formation from M1. The formation rate was proportional to incubation time up to 60 min at a protein concentration of 0.5 mg/ml. In all experiments, sibutramine, M1, and M2 were dissolved and serially diluted in methanol to the required concentrations; the solvent was subsequently evaporated under reduced pressure using an AES2010 SpeedVac (Thermo Electron, Waltham, MA). The incubation mixtures, which contained 10 µl of microsomes (5 mg protein/ml stock form three different human liver microsomal preparations) and various concentrations of sibutramine or M1 (0, 0.5, 1, 2, 5, 10, 20, 50, 100, 200, and 500 µM) were reconstituted in 100 mM phosphate buffer, pH 7.4, and prewarmed for 5 min at 37°C. The reaction was initiated by adding an NADPH-regenerating system (final volume of 100 µl) and incubated for an additional 5 min at 37°C in a shaking water bath. The reaction was terminated by placing the incubation tubes on ice and adding a 100-µl aliquot of acetonitrile solution containing 2.5 µg/ml propranolol (internal standard). After vortexing, the mixture was centrifuged at 16,000g for 10 min at 4°C, and 5-µl aliquots of the supernatant were injected into a liquid chromatography/tandem mass spectrometry (LC/MS/MS) system.

Chemical Inhibition Studies with HLMs. P450 isoform-selective inhibitors were used to determine the P450 isoform(s) involved in the metabolism of sibutramine. Reaction mixtures were incubated in the presence or absence of selectivity inhibitors, and M1 and M2 formation rates from sibutramine (0.5 and 5 µM, respectively) and M2 formation rate from M1 (0.5 and 5 µM, respectively) were calculated (Kim et al., 2006; Lee et al., 2006; Yoon et al., 2007). The P450 isoform-selective inhibitors included furafylline (10 µM) for CYP1A2, coumarin (100 µM) for CYP2A6, ticlopidine (2 µM; Richter et al., 2004), clopidogrel (1 µM; Richter et al., 2004) or thio-TEPA (5 µM) for CYP2B6, quercetin (25 µM) for CYP2C8, sulfaphenazole (10 µM) for CYP2C9, S-benzylmirtazapine (1 µM) for CYP2C19, quinidine (10 µM) for CYP2D6, diethyldithiocarbamate (10 µM) for CYP2E1, and ketoconazole (1 µM) for CYP3A. Except for the addition of selectivity inhibitors, all other incubation conditions were similar to those previously described (Shin et al., 1999, 2002). Furafylline, diethyldithiocarbamate, clopidogrel, ticlopidine, and thio-TEPA were preincubated for 15 min with HLMs and an NADPH-generating system before the addition of sibutramine or M1 to initiate the reaction. Other procedures were similar to those of the metabolism in HLMs. Activities of all the inhibitors were compared with those of respective inhibitor-free controls.

FIG. 1. Proposed metabolic pathway of sibutramine in human liver microsomes.
Clopidogrel-Induced Inactivation of Sibutramine Metabolism in HLMs. Kinetic inhibition studies of CYP2B6 were performed to determine the $K_i$ for clopidogrel during M1 formation using the method previously described by Obach et al. (2007) and Walsky and Obach (2007). An NADPH-generating system was incubated with various concentrations of clopidogrel (0, 0.1, 0.2, 0.5, 1, and 2 µM). The reaction mixture was prewarmed at 37°C for 3 min prior to the addition of HLMs, and the final reaction concentration was 5 mg protein/ml. At 0, 3, 5, 10, 15, and 20 min of incubation, a 20-µl aliquot was collected from each incubation mixture and transferred into a prewarmed secondary reaction mixture (37°C) containing 50 µM sibutramine and an NADPH-generating system in 0.1 M potassium phosphate buffer, pH 7.4, in a final volume of 200 µl. After 5 min of incubation, the reaction was terminated as described above. To determine the inactivation constants ($k_{inh}$ and $K_i$) that define concentration- and time-dependent inhibition, the relative inhibition of M1 formation from sibutramine was expressed as a percentage of the untreated time-matched control samples. Incubations carried out at time 0 were designated controls (100%). This procedure accounts for any loss of enzyme activity unrelated to the inhibitor. The natural logarithm of the mean relative inhibition values was plotted versus preincubation time for each concentration of inhibitor used. After visual inspection of the inactivation data (Ln% activity compared with the control) with preincubation time, the initial slope ($k_{inh}$) was determined using regression analysis. Initially, the parameters $k_{inh}$ and $K_i$ were obtained from the nonlinear fitting of $k_{inh}$ against the inhibitor concentration, assuming that the [I] remained constant. $k_{inh}$ is the theoretical maximum inactivation rate constant at [I] = 0, and $K_i$ is the inactivator concentration yielding 0.5 × $k_{inh}$.

Correlation Experiments. Sibutramine (5 µM) and M1 (5 µM) were incubated with microsomes from 16 different livers to examine the formation of M1 and M2 from sibutramine and M2 from M1, relative to specific P450 activity. The activity of each P450 isoform was determined using LC/MS/MS as previously described (Kim et al., 2005). Isoform-specific reaction markers were used to determine the activity of each P450, including phenacetin $O$-deethylation at 50 µM phenacetin (CYP1A2), coumarin 7-hydroxylation at 5 µM coumarin (CYP2A6), buproprion hydroxylation at 50 µM buproprion (CYP2B6), paclitaxel 6a-hydroxylation at 10 µM paclitaxel (CYP2C8), tolbutamide 4-methyldihydroxylation at 100 µM tolbutamide (CYP2C9), $S$-mephenytoin 4-hydroxylation at 100 µM S-mephenytoin (CYP2C19), dextromethorphan O-demethylation at 5 µM dextromethorphan (CYP2D6), chloroxazone 6-hydroxylation at 50 µM chloroxazone (CYP2E1), midazolam 1′-hydroxylation at 50 µM midazolam (CYP3A), and testosterone $β$-hydroxylation at 75 µM testosterone (CYP3A). The correlation coefficients between the formation rates for M1 and M2 from sibutramine or M2 from M1 and the activity of each P450 isoform were calculated by parametric regression analysis (SAS version 8.01; SAS Institute, Cary, NC). A p value less than 0.05 was considered statistically significant.

Metabolism of Sibutramine and M1 by cDNA-Expressed P450 Isoforms. To further identify the specific P450 isoforms involving in the M1 and M2 formation, different 10 cDNA-expressed P450s (CYP1A2, 2A6, 2B6, 2C8, 2C9, 2C19, 2D6, 2E1, 3A4, and 3A5; diluted to 200 pmol/ml with 100 mM phosphate buffer, pH 7.4) were incubated with an NADPH-generating system and sibutramine or M1 (both 0.5 and 5 µM) at 37°C for 5 min.

Full kinetic analyses were performed for the recombinant P450s that catalyzed sibutramine or M1. Sibutramine or M1 (0–500 µM) were incubated with 10 µl (200 pmol/ml) of cDNA-expressed CYP3A4, 3A5, 2B6, or 2C19 (sibutramine only), respectively. Other procedures were similar to those of the metabolism in HLMs.

LC/MS/MS Analysis of Sibutramine, M1, and M2. The concentrations of sibutramine, M1, and M2 in the above samples were analyzed by developed LC/MS/MS method in our laboratory. For the identification of sibutramine, M1, and M2, a tandem quadrupole mass spectrometer (QTrap 4000 LC/MS/MS; Applied Biosystems, Foster City, CA), coupled with an Agilent 1100 series high-performance liquid chromatography system (Agilent Technologies, Palo Alto, CA), was used. The separation was performed on a reversed-phase column (Luna phenyl-hexyl, 100–× 2.0-mm i.d.; 3-µm particle size; Phenomenex, Torrance, CA) using the mobile phase that consisted of acetonitrile and water containing 0.1% formic acid (47:53, v/v) at a flow rate of 0.2 ml/min. The column temperature was 40°C. The mass spectra were recorded by electrospray ionization with a positive ion mode. The turbo ion spray interface was operated at 5500 V and 400°C. The operating conditions were optimized by flow injection of analytes and were determined as follows: nebulizing gas flow, 40 psi; curtain gas flow, 10 psi; and collision gas (nitrogen) pressure, 3.58 × 10^{-5} Torr. Quadrupoles Q1 and Q3 were set on unit resolution.

Multiple reaction monitoring mode using specific precursor/product ion transition was used for the quantification. Detection of the ions was performed by monitoring the transitions of m/z 298.2 → 125.1 for sibutramine (collision energy, 31 eV), 266.5 → 125.1 for M1 (collision energy, 31 eV), 252.5 → 125.1 for M2 (collision energy, 31 eV), and 260 → 116 for propranolol (internal standard; collision energy, 25 eV), respectively. Peak areas for all the analytes were automatically integrated using Analyst software (version 1.4; Applied Biosystems). The lower limits of quantification for M1 and M2 were 20 nM, respectively. The intra- and interassay precision and accuracy for all the analytes were less than 15%.

Data Analysis. Results are expressed as mean ± S.D. of estimates obtained from three different liver microsomes or cDNA-expressed P450s isoforms in triplicate experiments. In the microsomal incubation studies, the apparent kinetic parameters of M1 and M2 from sibutramine and M2 from M1 were determined by fitting the unweighted kinetic data from HLMs and cDNA-expressed P450s to a single-site Michaelis-Menten equation [V = v_{max} · [I]/([I] + [K_i])], a sigmoidal (Hill) equation model [V = v_{max} · [S]/([K_i] + [S])] or, two-enzyme model [V = (v_{max1} · [S]/([K_i] + [S]) + v_{max2} · [S]/([K_i2] + [S]))]. The models that best fit were selected based on statistical goodness of fit (Yamaoka et al., 1978), and the model with the lowest Akaike information criterion was chosen. Calculated parameters were maximum rate of metabolic formation ($V_{max}$), Michaelis-Menten constant ($K_i$), intrinsic clearance ($CL_{int}$ = $V_{max}$/[$K_i$]), and Hill coefficient (n). Inactivation kinetic parameters were determined using nonlinear regression of the data from HLMs to the following equation: $k_{inh} = k_{inh0}([1] + a + k_{inh1} · [I]/[K_i + [I])]$. Calculations were performed using WinNonlin software (Pharsight, Mountain View, CA).

Results

Kinetic Analysis in HLMs. Kinetic analyses for M1 formation rate from sibutramine and M2 formation rate from M1 were performed using three separate HLM panels (Fig. 2, A and B). The estimated kinetics parameters are summarized in Table 1. The Eadie-Hofstee plot for M1 formation rate from sibutramine showed a biphasic pattern in three different HLM panels (Fig. 2A, inset). Accordingly, a two-enzyme model was used to estimate kinetic parameters, $V_{max}$ and $K_{mi}$, kinetic parameters for the high-affinity component, were calculated at 53.7 ± 31.8 pmol/min/mg protein and 4.79 ± 0.883 µM, respectively. $V_{max}$ and $K_{mi}$, kinetic parameters for the low-affinity component, were calculated at 897 ± 311 pmol/min/mg protein and 179 ± 10.9 µM, respectively. The in vitro intrinsic clearance ($CL_{int}$) for M1 formation from sibutramine was 10.8 ± 4.64 µl/min/mg protein in the high-affinity component and 4.96 ± 1.43 µl/min/mg protein in the low-affinity component. Because in vivo concentrations of sibutramine rarely exceed the $K_{mi}$ of the low-affinity component, the high-affinity process accounts for a large fraction of the total intrinsic clearance of sibutramine. Note that the calculated $CL_{int}$ values for M1 formation are clearly underestimated because they do not account for sequential metabolism from M1 to M2.

Similar to M1 formation from sibutramine, the Eadie-Hofstee plot for M2 formation from M1 showed a biphasic pattern (Fig. 2B, inset). $V_{max}$ and $K_{mi}$, kinetic parameters for the high-affinity component, were 163 ± 137 pmol/min/mg protein and 26.6 ± 19.2 µM, respectively. $V_{max}$ and $K_{mi}$, kinetic parameters for the low-affinity component, were 479 ± 60.0 pmol/min/mg protein and 348 ± 160 µM, respectively. The in vitro intrinsic clearance ($CL_{int}$) for M2 formation from M1 was 5.76 ± 0.992 µl/min/mg protein in the high-affinity component and 1.57 ± 0.896 µl/min/mg protein in the low-affinity component.

Correlation Analysis. The formation rates for M1 alone or both
M1 and M2 from sibutramine and the formation rate for M2 from M1 were compared against the activity of various P450 isoforms in 16 HLM panels. The corresponding values \( r \) and \( p \) were summarized in Table 2. The formation rates for both M1 from sibutramine and M2 from M1 were closely correlated with bupropion hydroxylation \( (r = 0.694, p = 0.0029 \text{ and } r = 0.834, p = 0.0001, \text{ respectively}; \text{Fig. 3, A and B}), indicating the involvement of CYP2B6. However, coumarin 7-hydroxylation (CYP2A6), \( S \)-mephenytoin 4-hydroxylation (CYP2C19), dextromethorphan O-demethylation (CYP2D6), midazolam 1'-hydroxylation (CYP3A), and testosterone 6β-hydroxylation (CYP3A) were not correlated with metabolite formation rates (Table 2). There was also a significant correlation between both M1 from sibutramine and M2 from M1 and bupropion hydroxylation \( (r = 0.718, p = 0.0017 \text{ and } r = 0.766, p = 0.0005, \text{ respectively}; \text{Table 2). However, rCYP2E1 was not capable of forming M1 and M2 (Figs. 4 and 6), and the formation of M1 and M2 formation is not inhibited by diethyldithiocarbamate, a specific inhibitor of CYP2E1. This may be linked to a close correlation between CYP2E1 and CYP2B6 activities \( (r = 0.759, p = 0.0006, \text{Supplemental Fig. 1)) \) in the panel of liver microsomes. Therefore, CYP2E1 does not appear to be involved in the formation of sibutramine active metabolites, M1 and M2. Three P450 isoforms (CYP1A2, CYP2C8, and CYP2C9) appeared to be associated with sibutramine metabolite formation (Table 2), although they also showed some correlation with bupropion hydroxylation, CYP2B6 (Supplemental Fig. 1).

Metabolism of Sibutramine by Human cDNA-Expressed P450s.

The formation rates of M1 and M2 from sibutramine \( (0.5, 5, \text{and } 200 \mu M, \text{respectively}) \) using human cDNA-expressed CYP1A2, 2A6, 2B6, 2C8, 2C9, 2C19, 2D6, 2E1, 3A4, and 3A5 are shown in Fig. 4. With the exception of CYP2B6, none of these P450s catalyzed the formation of M2 from sibutramine (Fig. 4, A and B). CYP2B6 catalyzed the conversion of sibutramine to M1 at the highest rate at 0.5 and 5 \( \mu M \). Although CYP2C19 and 3A4 also showed catalytic activity at a concentration of 0.5 \( \mu M \) sibutramine, metabolite formation rates were relatively slow (33.7 and 38.3% of CYP2B6 catalytic activity, respectively). In CYP3A5, the M1 formation was not observed. The corresponding values in CYP2C19, 3A4, and 3A5 at 5 \( \mu M \) sibutramine were 20.7, 17.5, and 38.3% of CYP2B6 catalytic activity, respectively. With the exception of CYP2B6, none of these P450s catalyzed the formation of M2 from sibutramine (Fig. 4C). This suggested the possibilities for sibutramine metabolism by the low-capacity system of CYP2B6 at higher concentration.

Therefore, we performed full kinetic analyses for the formation of M1 from sibutramine \( (0–500 \mu M) \) using human cDNA-expressed

![Fig. 2](https://example.com/fig2.png) Kinetics for the formation rate of M1 from sibutramine (A) and M2 from M1 (B) in different HLMs (\( n = 3 \)). An increasing concentration of sibutramine or M1 (0–500 \( \mu M \)) was incubated with HLMs (0.5 mg/ml) and an NADPH-generating system at 37°C for 5 min. The kinetic data were fit to a two-enzyme model. The respective corresponding Eadie-Hofstee plots are shown in the inset, respectively. Data are shown as mean ± S.D. V, formation rate of M1 (A) or M2 (B) (picomoles per minute per milligram of protein); S, concentration of sibutramine (A) or M1 (B) (micromolar).

### TABLE 1

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Substrate Sibutramine &amp; Metabolite M1</th>
<th>Substrate M1 &amp; Metabolite M2</th>
</tr>
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<tr>
<td>( V_{\text{max},1} ) (pmol/min/mg protein)</td>
<td>53.7 ± 31.8</td>
<td>163 ± 137</td>
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<tr>
<td>( K_{m,1} ) (( \mu )M)</td>
<td>4.79 ± 0.888</td>
<td>26.6 ± 19.2</td>
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<tr>
<td>( CL_{\text{int},1} ) (( \mu )l/min/mg protein)</td>
<td>10.8 ± 4.64</td>
<td>5.76 ± 0.992</td>
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<tr>
<td>( V_{\text{max},2} ) (pmol/min/mg protein)</td>
<td>897 ± 311</td>
<td>476 ± 60.0</td>
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<tr>
<td>( K_{m,2} ) (( \mu )M)</td>
<td>179 ± 10.9</td>
<td>348 ± 160</td>
</tr>
<tr>
<td>( CL_{\text{int},2} ) (( \mu )l/min/mg protein)</td>
<td>4.96 ± 1.43</td>
<td>1.57 ± 0.896</td>
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<tr>
<td>Total ( CL_{\text{int}} )</td>
<td>5.11 ± 1.53</td>
<td>1.87 ± 0.689</td>
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1682 BAE ET AL.
CYP2B6, 2C19, 3A4, and 3A5 (Fig. 5A; Table 3). For recombinant CYP2B6 and 3A4, the rate of M1 formation versus sibutramine concentration showed a better fit to a single-site Michaelis-Menten equation under our experimental conditions. For recombinant CYP2C19 and 3A5, the M1 formation rate versus sibutramine concentration was characterized by a sigmoidal curve, showing a better fit to a Hill equation. The Eadie-Hofstee plots in recombinant CYP2C19 and 3A5 showed concave and convex relationships, including negative ($n_{Hofstee}/H<0$) and positive ($n_{Hofstee}/H>0$) cooperativity, respectively (Fig. 5B). Although the $V_{max}$ values for formation rate of M1 by CYP3A4 and 3A5 were greater (1.92- and 4.77-fold, respectively) compared with that of CYP2B6, the $K_{m}$ values obtained from CYP2C19, 3A4, and 3A5 were much larger (48.4-, 10.6-, and 48.9-fold, respectively) than that of CYP2B6. Accordingly, the in vitro

**TABLE 2**

<table>
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<tr>
<th>Activity</th>
<th>P450 Isoforms</th>
<th>Correlation Coefficient ($r$)</th>
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<tr>
<td>Sibutramine (substrate), M1 (metabolite)</td>
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<tr>
<td>Phenacetin $O$-deethylation</td>
<td>CYP1A2</td>
<td>0.615 ($p = 0.011$)</td>
</tr>
<tr>
<td>Coumarin 7-hydroxylation</td>
<td>CYP2A6</td>
<td>0.323 ($p = 0.22$)</td>
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<tr>
<td>Bupropion hydroxylation</td>
<td>CYP2B6</td>
<td>0.694 ($p = 0.0029$)</td>
</tr>
<tr>
<td>Paclitaxel 6$\alpha$-hydroxulation</td>
<td>CYP2C8</td>
<td>0.657 ($p = 0.0057$)</td>
</tr>
<tr>
<td>Tolbutamide 4$\alpha$-hydroxylation</td>
<td>CYP2C9</td>
<td>0.583 ($p = 0.018$)</td>
</tr>
<tr>
<td>$S$-mephenytoin 4$\alpha$-hydroxylation</td>
<td>CYP2C19</td>
<td>0.0906 ($p = 0.74$)</td>
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<tr>
<td>Dextromethorphan $O$-demethylation</td>
<td>CYP2D6</td>
<td>0.351 ($p = 0.18$)</td>
</tr>
<tr>
<td>Chlorzoxazone 6$\beta$-hydroxylation</td>
<td>CYP2E1</td>
<td>0.715 ($p = 0.0018$)</td>
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<tr>
<td>Midazolam 1$\beta$-hydroxylation</td>
<td>CYP3A</td>
<td>0.342 ($p = 0.19$)</td>
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<td>Testosterone 6$\beta$-hydroxylation</td>
<td>CYP3A</td>
<td>0.107 ($p = 0.74$)</td>
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<td>M1 (substrate), M2 (metabolite)</td>
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</table>

**FIG. 3.** Correlation analyses between bupropion hydroxylation and the formation of M1 from sibutramine (A) and M2 from M1 (B) in 16 HLMs. Concentrations of sibutramine and M1 were both 5 $\mu$M.

**FIG. 4.** Representative plots of the formation of M1 and M2 from sibutramine by cDNA-expressed P450 isoforms. Human cDNA-expressed CYP1A2, 2A6, 2B6, 2C8, 2C9, 2C19, 2D6, 2E1, 3A4, and 3A5 were incubated with 0.5 (A), 5 (B), and 200 (C) $\mu$M sibutramine, respectively, at 37°C for 5 min. Data are shown as mean ± S.D. (picomoles per minute per picomole of P450) of triplicate experiments on 2 different days.

CYP2B6, 2C19, 3A4, and 3A5 (Fig. 5A; Table 3). For recombinant CYP2B6 and 3A4, the rate of M1 formation versus sibutramine concentration showed a better fit to a single-site Michaelis-Menten equation under our experimental conditions. For recombinant CYP2C19 and 3A5, the M1 formation rate versus sibutramine concentration was characterized by a sigmoidal curve, showing a better fit to a Hill equation. The Eadie-Hofstee plots in recombinant CYP2C19 and 3A5 showed concave and convex relationships, including negative ($n_{Hofstee}/H<0$) and positive ($n_{Hofstee}/H>0$) cooperativity, respectively (Fig. 5B). Although the $V_{max}$ values for formation rate of M1 by CYP3A4 and 3A5 were greater (1.92- and 4.77-fold, respectively) compared with that of CYP2B6, the $K_{m}$ values obtained from CYP2C19, 3A4, and 3A5 were much larger (48.4-, 10.6-, and 48.9-fold, respectively) than that of CYP2B6. Accordingly, the in vitro
by CYP2B6. After correcting for sequential metabolism to M2, the 
\( \text{CL}_{\text{int}} \) and \( K_m \) for CYP2B6-catalyzed M1 formation may be faster and smaller, respectively, than the listed values.

Next, we examined the rate of M2 formation from M1 (both 0.5 and 5 \( \mu \text{M} \)) using human cDNA-expressed CYP1A2, 2A6, 2B6, 2C8, 2C9, 2C19, 2D6, 2E1, 3A4, and 3A5. As shown in Fig. 6, A and B, CYP2B6 catalyzed the formation of M2 from M1 at the highest rate. Although CYP2C19, 3A4, and 3A5 also showed some activity at a high concentration of M1 (5 \( \mu \text{M} \)), these values were significantly lower compared with CYP2B6 (Fig. 6, A and B). Full kinetic analyses for the formation of M2 from M1 (0–500 \( \mu \text{M} \)) using human cDNA-expressed CYP2B6, 2C19, 3A4, and 3A5 are shown in Fig. 7. Under our experimental conditions, the rate of M2 formation versus M1 concentration showed a similar pattern to sibutramine metabolism.

The respective kinetic parameters derived from fitting the data to a single-site Michaelis-Menten equation for CYP2B6, 2C19, 3A4 and to a Hill equation for CYP3A5 are summarized in Table 4. The \( V_{\text{max}} \) values for the formation of M2 from M1 by CYP2B6 were 8.21-, 2.98-, and 2.46-fold larger than those of CYP2C19, 3A4, and 3A5, respectively, and the \( K_m \) values were 2.15-, 5.11-, and 4.22-fold smaller, respectively, than those of CYP2C19, 3A4, and 3A5. Consistent with these data, the in vitro \( \text{CL}_{\text{int}} \) for the CYP2B6-catalyzed formation of M2 from M1 was 17.5-, 15.2-, and 10.4-fold faster, respectively, than the corresponding values for CYP2C19, 3A4, and 3A5 (Table 4).

Chemical Inhibition of Sibutramine Metabolism. To further probe the role of P450 isoforms in sibutramine metabolism, sibutramine or M1 (both 0.5 and 5 \( \mu \text{M} \)) was incubated with P450 isoform-specific inhibitors in HLMs. At 0.5 \( \mu \text{M} \) sibutramine, clopidogrel (1 \( \mu \text{M} \)), ticlopidine (2 \( \mu \text{M} \)), and thio-TEPA (5 \( \mu \text{M} \)) inhibited M1 formation up to 61.9, 65.9, and 49.6% of the untreated control, respectively. At 5 \( \mu \text{M} \) sibutramine, the corresponding values were 56.1, 72.9, and 33.2% of the control, respectively. However, the same inhibitors had a much more significant effect on M2 formation when sibutramine was used as the substrate (0.5 \( \mu \text{M} \) sibutramine, up to 95.7, 90.9, and 69.5% of the control; 5 \( \mu \text{M} \) sibutramine, up to 93.9, 79.2, and 57.8% of the control; Fig. 8C). The effects of other inhibitors on the formation of M2 from sibutramine are not shown. Clopidogrel, ticlopidine, and thio-TEPA also potently inhibited the formation of M2 from 0.5 \( \mu \text{M} \) M1 up to 74.9, 89.6, and 61.2% of the control, respectively. At 5 \( \mu \text{M} \) M1, the corresponding values were 68.3, 84.9, and 55.6%, respectively. These results show that M2 formation is more susceptible to mechanism-based inhibitors of CYP2B6 than M1 formation. The effects of other inhibitors tested on the M1 formation from sibutramine and M2 formation from M1 were not shown (Figs. 7B and 8A).

Clopidogrel-Induced Inactivation of Sibutramine Metabolism. Walsky and Obach (2007) demonstrated that clopidogrel is the most potent mechanism-based inactivator of CYP2B6. To determine whether sibutramine metabolism is inhibited by clopidogrel, we carried out time- and concentration-dependent inactivation assays in HLMs. In the presence of clopidogrel, M1 formation decreased with increasing preincubation time in a dose-dependent manner (Fig. 9A).

The \( k_{\text{obs}} \) values were plotted against clopidogrel concentration and used to estimate maximal rates (\( k_{\text{inact}} \)) and \( K_i \) values in HLMs (Fig. 9B). The estimated maximal rates (\( k_{\text{inact}} \)) and apparent \( K_i \) values were 0.0368 min\(^{-1}\) and 0.206 \( \mu \text{M} \) in HLMs, respectively.

**Discussion**

We identified CYP2B6 as the major enzyme responsible for the sequential metabolism of sibutramine into M1 and then to M2 (Fig. 1). First, the formation of M1 and M2 from sibutramine and M2 from M1
were potently inhibited by well known CYP2B6 inhibitors, clopidogrel, ticlopidine, and thio-TEPA (Fig. 8, A–C). Recently, Kim and Liu (2007) reported that Woohwangcheongsimwon suspension, one of the most widely used traditional Chinese medicines for the emergency and acute treatment of stroke, numbness, hypertension, epilepsy, and arteriosclerosis, was a potent inhibitor of CYP2B6-mediated bupropion hydroxylation activity. In addition, Woohwangcheongsimwon suspension markedly inhibited the formation of M1 and M2 from 0.5 μM sibutramine and the formation of M2 from M1 by 45 and 57% of the untreated control, respectively (data not shown). Second, we observed a good correlation between the rate of M1 and M2 formation from sibutramine and bupropion hydroxylation (CYP2B6) activity and between M2 formation from M1 and bupropion hydroxylation activity, in 16 separate HLM panels (Table 2; Fig. 3A). When the combined formation rates of M1 and M2 were analyzed ($r = 0.727$, $p = 0.0014$, data not shown), this association with bupropion hydroxylation (CYP2B6) was stronger than that with M1 formation alone ($r = 0.694$, $p = 0.0029$; Table 2), suggesting that CYP2B6 catalyzes both metabolic steps. Finally, recombinant CYP2B6 produced M1 and M2 from sibutramine (Fig. 4, A and B) and M1 from M2 (Fig. 6, A and B) at a higher rate than other recombinant enzymes. Although CYP2C19, 3A4, and 3A5 also showed modest catalytic activity (Fig. 4, A and B), their contribution appears to be minor compared with CYP2B6. In addition, neither S-benzylirvanol (a CYP2C19-specific inhibitor) nor ketoconazole (a CYP3A family-specific inhibitor) inhibited the formation of M1 from sibutramine or the formation of M2 from M1 in HLMs. Similarly, the rates of M1 and M2 formation were not associated with S-mephenytoin 4-hydroxylation (CYP2C19 activity) or midazolam 1'-hydroxylation (CYP3A activity) in HLMs (Table 2). The in vitro CLint for CYP2B6-catalyzed M1 formation from sibutramine was 59.2-, 5.68-, and 10.5-fold faster

### Table 3

<table>
<thead>
<tr>
<th>Kinetic Parameters</th>
<th>CYP2B6</th>
<th>CYP2C19</th>
<th>CYP3A4</th>
<th>CYP3A5</th>
</tr>
</thead>
<tbody>
<tr>
<td>$V_{max}$ (pmol/min/pmol P450)</td>
<td>5.53 ± 0.0317</td>
<td>4.54 ± 0.0130</td>
<td>10.6 ± 0.321</td>
<td>26.4 ± 1.01</td>
</tr>
<tr>
<td>$K_m$ (μM)</td>
<td>8.02 ± 1.63</td>
<td>388 ± 83.7</td>
<td>84.7 ± 3.66</td>
<td>392 ± 20.7</td>
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<tr>
<td>$n$ (Hill coefficient)</td>
<td>0.710 ± 0.154</td>
<td>0.0120 ± 0.00262</td>
<td>0.125 ± 0.00163</td>
<td>0.0675 ± 0.000983</td>
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<tr>
<td>CLint ($μl/min/pmol P450$)</td>
<td>0.120 ± 0.000983</td>
<td>1.35</td>
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</table>

Fig. 6. Representative plots of the formation of M2 from M1 by cDNA-expressed P450 isoforms. Human cDNA-expressed CYP1A2, 2A6, 2B6, 2C8, 2C9, 2C19, 2D6, 2E1, 3A4, and 3A5 were incubated with 0.5 (A) and 5 (B) μM M1, respectively, at 37°C for 5 min. Data are shown as mean ± S.D. (picomoles per minute per picomole of P450) of triplicate experiments on 2 different days.

Fig. 7. Kinetics for the formation of M2 from M1 in human cDNA-expressed P450s. An increasing concentration of sibutramine (0–500 μM) was incubated with recombinant human CYP2B6 ( ), 2C19 ( ), 3A4 ( ), and 3A5 ( ) (20 pmol/ml each recombinant P450s) and an NADPH-generating system at 37°C for 5 min. The velocity (picomoles per minute per picomole of P450s) versus sibutramine concentration plot is shown in the inset.

![Graph](image-url)
than the corresponding values for CYP2C19, 3A4, and 3A5, respectively (Table 3). The CLint value for M2 formation from M1 was also 15.2- and 10.4-fold faster than the corresponding values for CYP3A4 and 3A5, respectively (Table 4). Based on these results, we concluded that a member or members of the human liver microsomal CYP3A subfamily and CYP2C19 play a minor role in sibutramine metabolism to M1 and then to M2.

In healthy men, the maximum plasma concentrations (Cmax) of sibutramine, M1, and M2 vary from 11 to 23, 8.05 to 26.8, and 30.4 to 41.7 nM, respectively, following a single oral administration of 15 mg of sibutramine (Hind et al., 1999; Abolfathi et al., 2004; Park et al., 2004; Jain et al., 2006). These values are similar to the in vitro range of substrate concentrations in our experiments, which indicated that CYP2B6 is the most powerful catalyst in stepwise sibutramine metabolism. Although recombinant CYP2C19, 3A4, and 3A5 also catalyzed M1 formation at high substrate concentrations (≥5 μM), CYP2B6 is capable of catalyzing both M1 and M2 formation at lower and more clinically relevant concentrations. When 100 nM sibutra-

![FIG. 8. Effects of P450 isomeric-selective inhibitors on the formation of M1 from sibutramine (A) and M2 from M1 (B) by HLMs. Effects of P450 2B6 inhibitors on the formation of M2 from sibutramine (C) by HLMs. Pooled HLMs (0.5 mg/ml) were incubated with 0.5 (■) and 5 (□) μM sibutramine and M1, respectively, in the absence (control) or presence of various chemical inhibitors at 37°C for 5 min. Data are presented as mean ± S.D. of triplicate experiments. FF, furafylline (10 μM) for CYP1A2; COU, coumarin (100 μM) for CYP2A6; TIC, ticlopidine (2 μM), CLO, clopidogrel (1 μM), and TEPA, thio-TEPA (5 μM) for CYP2C8; SUL, sulfaphenazole (10 μM) for CYP2C9; BEN, S-benzylisoyvanol (1 μM) for CYP2C19; QUE, quercetin (25 μM) for CYP2C19; QUIN, quinidine (10 μM) for CYP3A4; DETC, diethylidithiocarbamate (10 μM) for CYP2E1; KETO, ketoconazole (1 μM) for CYP3A.

### TABLE 4

<table>
<thead>
<tr>
<th>Kinetic Parameters</th>
<th>CYP2B6</th>
<th>CYP2C19</th>
<th>CYP3A4</th>
<th>CYP3A5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vmax (pmol/min/pmol P450)</td>
<td>15.1 ± 0.279</td>
<td>1.84 ± 0.182</td>
<td>5.06 ± 0.524</td>
<td>6.15 ± 0.131</td>
</tr>
<tr>
<td>Km (μM)</td>
<td>52.1 ± 3.04</td>
<td>112 ± 25.0</td>
<td>266 ± 33.0</td>
<td>220 ± 4.48</td>
</tr>
<tr>
<td>n (Hill coefficient)</td>
<td>1.40</td>
<td>1.40</td>
<td>1.40</td>
<td>1.40</td>
</tr>
<tr>
<td>CLint (μl/min/pmol P450)</td>
<td>0.291 ± 0.0230</td>
<td>0.0166 ± 0.00202</td>
<td>0.0192 ± 0.00142</td>
<td>0.0280 ± 0.000252</td>
</tr>
</tbody>
</table>
It is well known that CYP2B6 polymorphism is common in whites and that CYP2B6 is one of a highly polymorphic human P450s. Recent studies have revealed several new genetic variants of CYP2B6 (Lang et al., 2001; Lamba et al., 2003; Hesse et al., 2004; Lang et al., 2004), and this variability may play a major role in an individual’s ability to metabolize various endogenous and exogenous substrates (Turpeinen et al., 2006; Hodgson and Rose, 2007; Zanger et al., 2007). Previous studies have demonstrated that CYP2B6 polymorphism affects the metabolism and pharmacokinetics of bupropion (Kirchheiner et al., 2003), efavirenz (Desta et al., 2007), and cyclophosphamide (Xie et al., 2003; Nakajima et al., 2007). In addition, CYP2B6 expression or function is readily affected by exposure to inducer/inhibitor drugs and plays an important role in the metabolism of environmental chemicals (Hodgson and Rose, 2007).

Taken together, our in vitro data strongly support that CYP2B6 is a main catalyst in the sequential metabolism of sibutramine into M1 and M2 (Fig. 1). It would be very useful to understand the interindividual variability in pharmacokinetics of sibutramine and its metabolites and to predict drug-drug interactions. Moreover, the formation of M1 and/or M2 from sibutramine or M2 formation from M1 may prove useful as a specific marker of CYP2B6 activity at low substrate concentrations (<5 μM). Further study is required to determine whether CYP2B6-selective sibutramine N-demethylation is also observed in vivo.

**References**


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