The CYP2B6*6 Allele Significantly Alters the N-Demethylation of Ketamine Enantiomers In Vitro

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Received February 25, 2013; accepted April 2, 2013

ABSTRACT
Ketamine is primarily metabolized to norketamine by hepatic CYP2B6 and CYP3A4-mediated N-demethylation. However, the relative contribution from each enzyme remains controversial. The CYP2B6*6 allele is associated with reduced enzyme expression and activity that may lead to interindividual variability in ketamine metabolism. We examined the N-demethylation of individual ketamine enantiomers using human liver microsomes (HLMs) genotyped for the CYP2B6*6 allele, insect cell-expressed recombinant CYP2B6 and CYP3A4 enzymes, and COS-1 cell-expressed recombinant CYP2B6.1 and CYP2B6.6 protein variant. Effects of CYP-selective inhibitors on norketamine formation were also determined in HLMs. The two-enzyme Michaelis-Menten model best fitted the HLM kinetic data. The Michaelis-Menten constants (K_m) for the high-affinity enzyme and the low-affinity enzyme were similar to those for the expressed CYP2B6 and CYP3A4, respectively. The intrinsic clearance for both ketamine enantiomers by the high-affinity enzyme in HLMs with CYP2B6*1/*1 genotype were at least 2-fold and 6-fold higher, respectively, than those for CYP2B6*1/*6 genotype and CYP2B6*6/*6 genotype. The V_max and K_m values for CYP2B6.1 were approximately 160 and 70% of those for CYP2B6.6, respectively. N,N’-triethylenethiophosphoramide (thioTEPA) (CYP2B6 inhibitor, 25 μM) and the monoclonal antibody against CYP2B6 but not troleandomycin (CYP3A4 inhibitor, 25 μM) or the monoclonal antibody against CYP3A4 inhibited ketamine N-demethylation at clinically relevant concentrations. The degree of inhibition was significantly reduced in HLMs with the CYP2B6*6 allele (gene-dose P < 0.05). These results indicate a major role of CYP2B6 in ketamine N-demethylation in vitro and a significant impact of the CYP2B6*6 allele on enzyme-ketamine binding and catalytic activity.

Introduction
Ketamine [(RS)-2-(2-chlorophenyl)-2-(methylamino)cyclohexanone] is a noncompetitive N-methyl-b-aspartate receptor antagonist. It has been used as an anesthetic agent and an adjuvant analgesic in subanesthetic doses to attenuate opioid tolerance and opioid-induced hyperalgesia (White et al., 1982; Subramaniam et al., 2004). In addition, ketamine has displayed a rapid antidepressant effect and a preventive effect on postoperative interleukin 6 inflammatory response in clinical studies (Skolnick et al., 2009; Dale et al., 2012). However, these therapeutic applications of ketamine are frequently restricted by considerable interindividual variabilities in drug efficacy and undesired psychotomimetic effects (White et al., 1982; Meyer et al., 2004).

In most countries, ketamine is marketed as a racemic mixture consisting of equal amounts of (S)- and (R)-ketamine. (S)-Ketamine has an approximately 5-fold greater N-methyl-b-aspartate affinity and 4-fold greater analgesic potency, compared with (R)-ketamine, and is associated with less psychotomimetic effects (White et al., 1980; Mathisen et al., 1995, Ebert et al., 1997). Furthermore, the plasma clearance of (S)-ketamine is approximately 22% faster in vivo (White et al., 1985). However, no definitive evidence has been obtained from in vitro studies to support the stereoselective difference in ketamine clearance that is seen in vivo (Yanagihara et al., 2001; Portmann et al., 2010; Mossner et al., 2011). Ketamine is predominantly metabolized by hepatic cytochrome P450 (P450)–mediated N-demethylation to norketamine, a weakly active metabolite that has approximately one-third the anesthetic activity of its parent drug in rats (White et al., 1975). Several studies using microsomes containing cDNA-expressed human P450 enzymes have shown the involvement of CYP2B6, CYP2C9, CYP2C19, and CYP3A4 in ketamine N-demethylation. Among these CYP enzymes, CYP2B6 exhibited the highest demethylation activity, followed by CYP3A4 (Yanagihara et al., 2001; Hijazi and Boulic, 2002;
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Portmann et al., 2010. In human liver microsomes (HLMs), Yanagihara et al. reported that CYP2B6 is the major enzyme responsible for (S)- and (R)-ketamine N-demethylation (Yanagihara et al., 2001). However, this was not confirmed by later studies, which identified that the primary contribution was from CYP3A4 (Hijazi and Boulieu, 2002; Mossner et al., 2011).

CYP2B6 is responsible for the hepatic metabolism of several other clinically important drugs, including efavirenz, methadone, bupropion, propofol, and cyclophosphamide (Zanger et al., 2007; Turpeinen and Zanger, 2012). It exhibits substantial interindividual variability with regard to both its catalytic activity and level of expression, which can be partially explained by the genetic variability of the highly polymorphic CYP2B6 gene. Among the currently described 30 alleles (www.cypalleles.ki.se/cyp2b6.htm), the CYP2B6*6 allele is the most prevalent and clinically important variant. This variant is characterized as a haplotype consisting of two linked nonsynonymous single nucleotide polymorphisms (SNPs), c.516G>T (rs3745274) and c.785A>G (rs2279343), which reduce the expression of functional enzyme (Hofmann et al., 2008). The CYP2B6*6 allele has previously been associated with approximately 2-fold greater plasma concentration of efavirenz (Haas et al., 2004) and a 6-fold decrease in efavirenz 8-hydroxylation by HLMs (Xu et al., 2012). Of interest, the influence of the CYP2B6*6 allele on in vitro drug metabolism appears to be substrate dependent, with bupropion and efavirenz being negatively affected, and cyclophosphamide 4-hydroxylation activity was generally found to be higher for the variant (Xie et al., 2006; Ariyoshi et al., 2011; Xu et al., 2012). In contrast to these well-investigated CYP2B6 substrates, nothing is known regarding the impact of the CYP2B6*6 allele on ketamine N-demethylation.

The aim of this study was to evaluate the relative contribution of CYP2B6 and CYP3A4 to the N-demethylation of (S)- and (R)-ketamine using HLMs and expressed CYP2B6 and 3A4 isofoms and to assess the impact of CYP2B6*6 allelic variant on ketamine metabolism.

Materials and Methods

Chemicals. Boric acid, bromophenol blue, cyclopentylbromide, 2-chlorobenzonitrile, ethidium bromide, dimethyl sulfoxide BioReagent, ethylenediaminetetraacetic acid, N-isocitric acid, isocitric dehydrogenase, (R,S)-ketamine hydrochloride, (S)-ketamine hydrochloride, sodium acetate, sodium pyrophosphate, d(+)-tartaric acid, thiopETP (N,N’-triethylenephosphoramidate), and trizma base were purchased from Sigma-Aldrich (Castle Hill, NSW, Australia). Bovine serum albumin; Thermopol reaction buffer; Taq DNA polymerase; Bsu, BsvI, StyI, and SspI restriction enzymes; and corresponding reaction buffers were purchased from New England Biolabs (distributed by Genesearch, Arundel, QLD, Australia). Disodium hydrogen orthophosphate, dipotassium hydrogen orthophosphate, hydrochloric acid, magnesium chloride hexahydrate, sodium carbonate, and sodium dihydrogen orthophosphate were obtained from Ajax Chemicals (Auburn, NSW, Australia). NaDAP-sodium salt, potassium chloride, and trizma base were obtained from GeneWorks (Thebarton, SA, Australia). Acetonitrile, diethyl ether, hexane and sucrose were purchased from Chem-supply (Gillman, SA, Australia). Agarose I was purchased from AMRESCO (distributed by Astral Scientific, Gymea, NSW, Australia). N,N-diethyl-N-nitro-N-naphthylamine was from Applied Biosystems (Mulgrave, VIC, Australia). Butylated hydroxytoluene was from MP Biochemicals (Irvine, CA). Deoxyribonucleoside triphosphate was purchased from Finnzymes (distributed by Genesearch). BigDye version 3.0 sequencing reagents were obtained from Applied Biosystems (Mulgrave, VIC, Australia). Maxwell 16 tissue DNA purification kits were purchased from Promega (Madison, WI).

Preparation of (R,S)-Norketamine, (S)-Norketamine, and (R)-Ketamine. Racemic norketamine hydrochloride was prepared starting from 2-chlorobenzonitrile. Treatment with cyclopentylmagnesium bromide in the presence of copper (I) bromide, followed by hydrolysis, gave the cyclopentyl-(2-chlorophenyl) ketone (Weiberth and Hall, 1987). This was brominated with N-bromosuccinimide, followed by treatment with liquid ammonia to form an imine intermediate. Thermal rearrangement of the imine afforded (R,S)-norketamine (Fig. 1). The structures of these chemicals were confirmed by 1H and 13C NMR spectra with use of a Varian Gemini (300 MHz) instrument (Agilent Technologies, Mulgrave, VIC, Australia). NMR spectra were recorded in CDCl3 solution using tetramethylsilane (0 ppm) and CDCl3 (77.0 ppm) as internal standards for 1H and 13C, respectively. Optical resolution of the enantiomers was accomplished through formation of the diastereomeric tartrate salts (Hong and Davison, 1982). (R)-kemrine hydrochloride was separated from (S)-kemrine hydrochloride through a modification of the published procedure for the resolution of (S)-ketamine (Steiner et al., 2000).

Expressed Proteins and Monoclonal Antibodies. CYP2B6.1 and CYP2B6.6 protein variants were transiently expressed in COS-1 cells with use of pCMV-derived expression vectors, and microsomal fractions were prepared according to a previously described method (Lang et al., 2004). Human P450

Fig. 1. Scheme of (R,S)-norketamine synthesis. Intermediates: (1) cyclopentyl-(2-chlorophenyl) ketone, (2) α-Bromo-(2-chlorophenyl)cyclopentyl ketone, (3) 1-[(2-chlorophenyl)iminomethyl]cyclopentanol, and (4) (R,S)-norketamine hydrochloride. Reaction conditions: (A) magnesium, iodine crystals, anhydrous ether, 1 hour; (B) 2-chlorobenzonitrile, copper (I) bromide, anhydrous ether, 16 hours; (C) N-bromosuccinimide, p-toluensulfonic acid, dichloromethane, 6 hours; (D) liquid ammonia, â–ºC, 18 hours; and (E) isopropanol, reflux, 5 days.
isomers (CYP3A4 and CYP2B6) coexpressed with P450 reductase and cytochrome b5 (Cyt b5) in baculovirus-infected insect cells were commercially available from BD Gentest (North Ryde, NSW, Australia). Monoclonal antibodies inhibitory to human CYP3A4 and to human CYP2B6, antibodies against human CYP3A kit (WB-MAB-3A), and antibodies against human CYP2B6 kit (WB-2B6-PFP) for immunoblotting were also obtained from BD Gentest.

Liver Samples. Ethical approval was obtained from the Committee on the Ethics of Human Experimentation of the University of Adelaide and Human Ethics Committee of the Royal Adelaide Hospital. Human liver samples were donated by 23 patients undergoing partial hepatectomy for hepatic tumors. All liver samples were genotyped for the major CYP2B6 alleles by assays described below, and 11 liver tissues were selected on the basis of their CYP2B6*6 genotype. Characteristics of the 11 patients were as follows: (1) all were white; (2) ages ranged from 31 to 77 years; (3) six of eleven were male; and (4) four patients carried CYP2B6*1/*1 genotype, four carried CYP2B6*1/*6 genotype, and three carried CYP2B6*6/*6 genotype; and (5) nine patients had normal clinical chemistry and hematology before surgery, one patient had high concentrations of lactate dehydrogenase (3.4 × upper limit of normal) and transaminases (8 × upper limit of normal), and one patient had high concentration of alanine transaminase (13.6 × upper limit of normal). All tissue samples were frozen in liquid nitrogen and stored at −80°C until use.

CYP2B6 Genotyping. Genomic DNA was isolated from liver tissues with use of Maxwell 16 instrument with Maxwell 16 Tissue DNA purification kit according to the manufacturer’s protocol. SNPs related to CYP2B6*5 (c.1459C>T) (Nakajima et al., 2001) and CYP2B6*6 (c.516G>T and c.785A>G), CYP2B6*7 (c.516G>T, c.785A>G and c.1459C>T), CYP2B6*8 (c.415A>G), CYP2B6*13 (c.415A>G, c.516G>T and c.785A>G), and CYP2B6*1*6 allele were screened using previously described polymerase chain reaction–restriction fragment length polymorphism assays (Lang et al., 2004; Nakajima et al., 2001). Genotypes of random samples were confirmed by DNA sequencing (BigDye, version 3.0).

Preparations of HLMs. HLMs were prepared using a previously described differential centrifugation method (Zanger et al., 1988). Total protein content of microsomes was measured using the carbon monoxide difference spectrum assay (Omura and Sato, 1964). The CYP2B6 and CYP3A contents were determined using quantitative Western blot analysis with use of WB-2B6-PFP or WB-MAB-3A kits according to the manufacturer’s protocol. The dilutions of primary (1st) and secondary (2nd) antibodies for the detection of CYP3A and CYP2B6 in HLMs were 1:1000/1:10000 and 1:1000/1:1000, respectively.

Enzyme Kinetics in HLMs. Norketamine formation from separate (S)- and (R)-ketamine in HLMs was performed as follows. All incubations were performed in duplicate at 37°C in a shaking water bath. Incubation medium, in a final volume of 200 μl, consisted of 100 μg/ml HLM proteins, NADPH-regenerating system (1 mM NADP, 1 unit/ml−1 isocitrate dehydrogenase, 5 mM t tartaric acid, 5 mM magnesium chloride), and 25 mM of disodium phosphate buffer (pH 7.4). After 3-minute preincubation, the reaction was initiated by the addition of substrate and terminated after 30 minutes incubation. Norketamine formation was quantified using the HPLC assay as described below. Inhibition Studies with Chemical Inhibitors. The effects of chemical inhibitors selective for CYP2B6 (thioTEPA, 25 μM) and CYP3A (troleandomycin, 25 μM) on the formation of (S)- and (R)-norketamine from both low (equivalent to respective Kₘ values for each genotype group) and high (equivalent to respective Kᵢ values for each genotype group) ketamine concentrations were studied in the 11 HLMs. The effects of chemical inhibitors at various final concentrations (5–100 μM) on norketamine formation were tested. The final concentration of each inhibitor (25 μM) was selected to optimize selective inhibition. Stock solutions of inhibitors were prepared in 0.1 M sodium phosphate buffer (pH 7.4) with 5% methanol. All components of the incubation medium except ketamine were preincubated with inhibitors or methanol control for 20 minutes at 37°C. The reaction was then initiated by the addition of substrate and terminated after 30 minutes incubation. Norketamine formation was quantified using the HPLC assay with UV detection that was modified from a previously reported method (Chong et al., 2009). In brief, separation of compounds was achieved in a 3-μm C8 reverse-phase column (150 × 4.6 mm; Luna; Phenomenex, Torrance, CA) with a mobile phase of 15% (v/v) acetonitrile and 0.05% triethylamine in 20 mM dipotassium phosphate (pH 3), and the flow rate was 0.8 ml/min. The column was protected by a guard cartridge system packed with 4 × 3 mm C8 SecurityGuard cartridge (Phenomenex). Analytes were detected at 210 nm. The peak area was calculated using Shimadzu Class VP software (version 6.12, SP2; Shimadzu, Kyoto, Japan). The retention times for ketamine, norketamine, and ephedrine were 11 minutes, 9 minutes, and 4 minutes, respectively. Norketamine formation was quantified using calibration curves consisting of eight standards of norketamine over the concentration range of 0.5–200 μM. Inter-assay and intra-assay variabilities were determined using the analysis of duplicates of quality-control norketamine samples at three different concentrations: low (2.5 μM), medium (25 μM), and high (80 μM). The inter-assay (n = 6) and intra-assay (n = 6) precision and inaccuracy of all quality-control samples were less than 10%. The precision and inaccuracy for the limit of quantification (n = 6) were below 10%.

Data Analysis. Eadie-Hofstee plots were used for analysis of enzyme kinetic data. The kinetic parameters (Kₘ and Vₘₐₓ) were estimated using one-enzyme Michaelis-Menten model and a user-defined two-enzyme Michaelis-Menten model (ν = Vₘₐₓ[S]/[Kₘ+S]+[S]+Vₘₐₓ[S]/[Kₘ+S]) with use of GraphPad Prism 5 software (San Diego, CA). Goodness of fit of data were compared between the two models. Intrinsic clearance (Cᵢₘₐₓ) was calculated as Vₘₐₓ/Kₘ.

Inhibition data were expressed as a percentage of the corresponding controls. The differences in P450 protein content, kinetic parameters, and inhibitory effects on norketamine formation rate among CYP2B6*6 genotypes were determined by Jonckheere-Terpstra test using SPSS, version 19 (IBM, Armonk, NY). Correlations between CYP2B6 or CYP3A4 content and maximal norketamine formation rate were assessed using Spearman’s rank correlation. Stereoselective differences in kinetic parameters and inhibitory effects were analyzed using two-tailed Wilcoxon matched-pairs signed rank test. Data are presented as median (range) (unless otherwise specified). The results were considered to be statistically significant when P < 0.05.
Results

CYP2B6 and CYP3A4 Protein Content. A significant reduction in CYP2B6 protein content was confirmed in HLMs with the CYP2B6*1/*6 (median, 18.9 pmol/mg protein; range, 13.0–32.0 pmol/mg protein; n = 4) and *6/*6 genotype (median, 10.4 pmol/mg protein; range, 8.1–12.2 pmol/mg protein; n = 3), compared with HLMs carrying the *1/*1 genotype (median, 23.4 pmol/mg protein; range, 21.2–37.6 pmol/mg protein; n = 4; Jonckheere-Terpstra test, P = 0.005). There was also approximately 5-fold variability in the CYP3A4 protein content among individual HLMs, but it was not correlated with CYP2B6*6 genotypes (P = 0.28).

Enzyme Kinetics in HLMs. Nonlinear Eadie-Hofstee plots indicated the participation of at least two enzymes in the N-demethylation of (S)- and (R)-ketamine in HLMs (Fig. 2). The two-enzyme Michaelis-Menten model provided a much better fit to the data, compared with the single-enzyme Michaelis-Menten model. The estimated values of kinetic parameters for the high-affinity/low-capacity enzyme (Vmax1, Km1, and CLint1) and the low-affinity/high-capacity enzyme (Vmax2, Km2, and CLint2) are listed in Table 1. The variability in kinetic parameters among three CYP2B6 genotypes is shown in Table 1 and Fig. 3. For (S)-ketamine, significant gene-dose effects in Km1 and CLint1 values among three genotype groups were identified (P = 0.03 and 0.008, respectively). For (R)-ketamine, gene-dose effects in Km1, CLint1, and CLint2 values were found (P = 0.008; P = 0.001; and P = 0.008, respectively).

The maximal formation rates (Vmax, pmol/min/mg protein) and intrinsic clearance (CLint, ml/min/mg protein) of norketamine enantiomers by the high-affinity/low-capacity enzyme were better correlated with the CYP2B6 content (Vmax: Spearman r = 0.7; P = 0.02; and r = 0.56; P = 0.08 for (S)- and (R)-norketamine, respectively; CLint1: r = 0.81; P = 0.004; and r = 0.80; P = 0.005 for (S)- and (R)-norketamine, respectively), compared with the CYP3A4 content (Vmax1: r = −0.05; P = 0.90; and r = 0.33; P = 0.33 for (S)- and (R)-norketamine, respectively; CLint1: r = 0.19; P = 0.57; and r = 0.37; P = 0.26 for (S)- and (R)-norketamine, respectively) (Figs. 4 and 5). Conversely, the maximal norketamine formation rates and intrinsic clearance by the low-affinity/high-capacity enzyme were better correlated with CYP3A4 content (Vmax1: r = 0.55; P = 0.09; and r = 0.39; P = 0.24 for (S)- and (R)-norketamine, respectively; CLint1: r = 0.71; P = 0.02; and r = 0.80; P = 0.005 for (S)- and (R)-norketamine, respectively) but not with CYP2B6 content (Vmax: r = 0.35; P = 0.30; and r = 0.00; P > 0.99 for (S)- and (R)-norketamine, respectively; CLint: r = 0.20; P = 0.56; and r = −0.04; P = 0.92 for (S)- and (R)-norketamine, respectively).

There were no significant differences between (S)- and (R)-ketamine N-demethylation observed for any kinetic parameters (Wilcoxon signed ranks test, P > 0.07).

Enzyme Kinetics in cDNA-Expressed P450 Enzymes and Expressed CYP2B6 Variants. Table 2 shows the kinetic parameters of metabolism of (S)- and (R)-ketamine to norketamine by baculovirus-infected insect cell microsomes containing cDNA-expressed CYP2B6 and CYP3A4 coexpression of Cyt b5 and by COS-1 cell-expressed CYP2B6.1 and CYP2B6.6 protein variants. The Km values for the expressed CYP2B6 were considerably lower than those for the expressed CYP3A4. The Vmax values were not substantially different among these three isoforms.

The mean Km value determined for (S)-ketamine N-demethylation by the insect cell–expressed CYP2B6 was significantly lower than that for (R)-ketamine (P = 0.03), and a greater mean CLint value was found for (S)-ketamine N-demethylation (P = 0.03), but not for the COS-1 cell–expressed CYP2B6.1 protein (P = 0.69). Differences in kinetic parameters were not apparent for the two enantiomers of ketamine with other expressed proteins (P > 0.05).

Inhibition Study. Figure 6 shows the effects of CYP2B6- and CYP3A3-specific chemical inhibitors and inhibitory monoclonal antibodies on the N-demethylation of ketamine at low and high concentrations in HLMs carrying the three different CYP2B6 genotypes. At low ketamine concentrations (equivalent to the corresponding mean Km1 values for each CYP2B6 genotype group in HLMs kinetic assay,

<table>
<thead>
<tr>
<th>Variable</th>
<th>Vmax1</th>
<th>Km1</th>
<th>CLint1</th>
<th>Vmax2</th>
<th>Km2</th>
<th>CLint2</th>
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<tr>
<td>(S)-Ketamine</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>*1/*1</td>
<td>3.9 (2.1–12)</td>
<td>28 (12–43)</td>
<td>226 (68–281)</td>
<td>31 (11–43)</td>
<td>471 (375–1673)</td>
<td>52 (22–79)</td>
</tr>
<tr>
<td>*1/*6</td>
<td>7.4 (5.3–9.4)</td>
<td>86 (63–139)</td>
<td>84 (51–121)</td>
<td>16 (5.7–20)</td>
<td>374 (213–532)</td>
<td>36 (27–51)</td>
</tr>
<tr>
<td>*6/*6</td>
<td>2.6 (1.9–4.7)</td>
<td>78* (71–85)</td>
<td>37* (24–56)</td>
<td>15 (12–26)</td>
<td>934 (638–969)</td>
<td>19 (16–27)</td>
</tr>
<tr>
<td>(R)-Ketamine</td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>*1/*1</td>
<td>3.3 (2.3–8.1)</td>
<td>20 (69–42)</td>
<td>228 (108–333)</td>
<td>21 (13–71)</td>
<td>339 (230–1475)</td>
<td>64 (32–82)</td>
</tr>
<tr>
<td>*1/*6</td>
<td>6.3 (4.6–9.5)</td>
<td>69 (52–98)</td>
<td>98 (71–105)</td>
<td>19 (15–23)</td>
<td>612 (400–825)</td>
<td>36 (18–46)</td>
</tr>
<tr>
<td>*6/*6</td>
<td>2.0 (1.9–4.1)</td>
<td>74 (66–91)</td>
<td>26 (22–61)</td>
<td>5.7 (4.9–23)</td>
<td>840 (621–1046)</td>
<td>8* (5.5–27)</td>
</tr>
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</table>

* Significant gene-dose effects among the three genotype groups, Jonckheere-Terpstra test, P < 0.05.
20–80 μM), the inhibitory effects of thioTEPA (25 μM) on norketamine formation in HLMs with CYP2B6*1/*1 genotype [67 and 62% for (S)- and (R)-norketamine, respectively] were significantly greater than those in HLMs with CYP2B6*1/*6 [39 and 35% for (S)- and (R)-norketamine, respectively] and CYP2B6*6/*6 genotypes [18% for both enantiomers; Jonckheere-Terpstra test, P = 0.007]. Troleandomycin (25 μM) significantly inhibited (S)- and (R)-norketamine formations only in HLMs with CYP2B6*1/*6 genotype [P = 0.002; and 0.007 for (S)- and (R)-norketamine, respectively], with median values of percentage of inhibition less than 6%. At high ketamine concentrations (equivalent to the corresponding K_m values, 300–850 μM), troleandomycin significantly diminished (S)- and (R)-norketamine formation by 30–46% (P < 0.0001); however, significant gene-dose effect on the degree of inhibition was not observed (Jonckheere-Terpstra test, P = 0.62). ThioTEPA had no effect on norketamine formation at these substrate concentrations [P = 0.38; and 0.50 for (S)- and (R)-norketamine, respectively].

MAB2B6 inhibited norketamine formation at low ketamine concentrations (P < 0.0001 for both enantiomers), with a higher median percentage inhibition in HLMs with CYP2B6*1/*1 [57 and 46% for (S)- and (R)-norketamine, respectively], compared with HLMs with CYP2B6*1/*6 [27 and 37% for (S)- and (R)-norketamine, respectively] and CYP2B6*6/*6 genotypes [19 and 30% for (S)- and (R)-norketamine, respectively]. MAB2B6 did not cause inhibition at high ketamine concentrations [P = 0.77; and 0.41 for (S)- and (R)-norketamine, respectively]. MAB3A4 inhibited norketamine formations by 30–50% at high substrate concentrations (P < 0.005), but it had no effect at low substrate concentrations. A significant gene-dose effect was not observed with MAB3A4 inhibition [P = 0.65; and 0.39 for (S)- and (R)-norketamine, respectively]. None of the four inhibitors exhibited stereoselective preference on norketamine formation at either substrate concentration.

Discussion
To our knowledge, the present study provides the first evidence that the CYP2B6*6 genetic variant has a major impact on the N-demethylation of ketamine to norketamine in vitro, which is likely to be attributable to impairment in both enzyme-substrate binding and catalytic activity. The estimated K_m values for (S)- and (R)-norketamine formation by the high-affinity/low-capacity enzyme and the low-affinity/high-capacity enzyme in HLMs with CYP2B6*1/*1 genotype were similar to those values for the insect cell-expressed CYP2B6 and CYP3A4, respectively. Therefore, the high-affinity/low-capacity enzyme very likely corresponds to CYP2B6 and the low-affinity/high-capacity to CYP3A4. This finding was also supported by the results of Spearman correlation analysis between the values of K_m or V_{max} and P450 protein expression. The K_m values for the high-affinity/low-capacity enzyme in HLMs with CYP2B6*1/*1 genotype were at least 3-fold and 2.7-fold lower, respectively, than those for HLMs with CYP2B6*1/*6 and CYP2B6*6/*6 genotypes. Similarly, significantly lower K_m values for the COS-1 cell-expressed CYP2B6.1 protein, compared with the CYP2B6.6 variant, were observed. These results suggest an influence of the CYP2B6*6 allele on enzyme-ketamine binding. In addition to the increase in K_m values, V_{max} values for (S)- and (R)-norketamine formation in CYP2B6.6 variant significantly decreased by 41 and 35%, respectively, compared with CYP2B6.1, despite the minor difference in CYP2B6 expression levels between the two proteins. Therefore, the genetic impact of the CYP2B6*6 allele on V_{max} values is likely to be attributable to an impairment in catalytic activity rather than in enzyme expression level. In human liver micromosomal assays, however, no gene-dose effect in V_{max} values was observed. This may be attributable to the low level of CYP2B6 as a percentage of total hepatic P450 content, and because these V_{max} values were normalized to total micromosomal P450 content, they might not accurately reflect the influence of the mutation in norketamine formation rate. As a consequence of the reduction in both enzyme-substrate binding and catalytic activity, intrinsic clearance rates of ketamine enantiomers by the high-affinity/low-capacity enzyme were decreased by at least 62% in HLMs with CYP2B6*1/*6 genotype and 84% in HLMs with CYP2B6*6/*6 genotype. This is consistent with the results for the COS-1 cell-expressed CYP2B6 protein variants. Surprisingly, a significant gene-dose effect of the CYP2B6*6 allele on the intrinsic clearance rates of (R)-ketamine by the low-affinity/high-capacity enzyme was observed, which is possibly attributable to a small sample size and large variability in CYP3A4.
The impact of the CYP2B6*6 allele on ketamine metabolism was further confirmed by inhibition assays, in which the inhibitory effect of thioTEPA and MAB2B6 on norketamine formation from low ketamine concentrations was substantially reduced in HLMs carrying CYP2B6*1/*6 (by 40–50%) and CYP2B6*6/*6 genotypes (by 60–70%), compared with HLMs with CYP2B6*1/*1 genotype.

The CYP2B6*6 allele has been previously associated with an increase in $K_m$ values and decrease in $V_{max}$ values for efavirenz 8-hydroxylation and bupropion 4-hydroxylation (Xu et al., 2012). In contrast, it has also been associated with decreased $K_m$ values for cyclophosphamide 4-hydroxylation (Ariyoshi et al., 2011). The substrate-dependent effects of the allele on enzyme-substrate binding and
CYP2D6, with the half maximal inhibitory concentration and the of other P450 isoforms, including CYP2B6, CYP2C9, CYP2C19, and discrepancy may be a result of ketoconazole also being a potent inhibitor formation (Yanagihara et al., 2001; Mossner et al., 2011). This dis-
bodies against CYP3A4 failed to produce any inhibition on norketamine 
trast, another CYP3A4 inhibitor cyclosporin A and monoclonal anti-
the greatest inhibitory effects on norketamine formation from 25 or 50 
concen-
trations is more likely to be CYP3A4.

In the two studies that described a major role of CYP3A4 in ketamine 
demethylation, the CYP3A4 inhibitor ketoconazole at 10 
metabolism, the CYP3A4 inhibitor ketoconazole at 10 

In conclusion, here, we demonstrate that the most common allelic 
variant of CYP2B6 gene, CYP2B6*6, is associated with the decrease in 
both enzyme ketamine binding and N-demethylation activity. To the 
best of our knowledge, this is the first report showing the impact of 
catalytic activity suggest that this influence of the CYP2B6*6 allele 
are more complicated than a decrease in enzyme expression. A previous 
report has shown that the c.516G>T polymorphism, one of the two 
nonsynonymous SNPs of the allele, induced high levels of an 
alternative splicing variant that may be responsible for altered 
enzyme activity (Hofmann et al., 2008). However, whether the 
distinct substrate-dependent genetic impact is caused by the c.516G>T 
polymorphism and its molecular mechanism needs to be further 
investigated.

Another major finding of the current study is that the human liver 
microsomal N-demethylation of ketamine, at clinically relevant 
concentrations, is predominantly mediated by CYP2B6. By comparison, 
CYP3A4 appears to be a dominant contributor at much higher 
substrate concentrations. At low ketamine concentrations (20–80 
μM) that are similar to the extrapolated peak hepatic concentration 
(approximately 50 μM) after an intravenous dose of 2 mg/kg of 
racemic ketamine for anesthesia (Hijazi and Boullieu, 2002), 
substantial inhibition of (S)- and (R)-norketamine formation by the 
CYP2B6 inhibitor thiOPEA and MAB2B6 but minor inhibition by 
troleandomycin and MAB3A4 were found, suggesting a major 
preference 
CYP2B6*6 allele may be useful in 

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TABLE 2

<table>
<thead>
<tr>
<th>Variable</th>
<th>(S)-Ketamine</th>
<th>(R)-Ketamine</th>
</tr>
</thead>
<tbody>
<tr>
<td>V_{max}</td>
<td>k_{m}</td>
<td>V_{max}</td>
</tr>
<tr>
<td>pmol/min/pmol CYP (P450)</td>
<td>μM</td>
<td>pmol/min/pmol CYP (P450)</td>
</tr>
<tr>
<td>Baculovirus-insect cell–expressed CYP (P450) isoforms with coexpression of Cyt b5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CYP2B6</td>
<td>88 (83–90)</td>
<td>51 (50–54)</td>
</tr>
<tr>
<td>CYP3A4</td>
<td>100 (99–132)</td>
<td>453 (324–949)</td>
</tr>
<tr>
<td>COS-1 cell–expressed CYP2B6 variants</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CYP2B6*1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>75 (68–86)</td>
<td>50 (38–63)</td>
</tr>
<tr>
<td>CYP2B6*6&lt;sup&gt;b&lt;/sup&gt;</td>
<td>47&lt;sup&gt;c&lt;/sup&gt; (45–49)</td>
<td>71&lt;sup&gt;c&lt;/sup&gt; (64–79)</td>
</tr>
</tbody>
</table>

<sup>a</sup> CYP2B6<sup>f</sup>: CYP2B6 wild type protein
<sup>b</sup> CYP2B6<sup>f</sup>: variant carrying amino acid changes Q172H and K262R.
<sup>c</sup> Significant difference compared with corresponding values for CYP2B6<sup>f</sup> group. Wilcoxon signed-rank test, P < 0.05.
<sup>d</sup> Significant difference compared with corresponding values for (S)-ketamine group. Wilcoxon signed-rank test, P < 0.05.

<sup>Δ</sup>b: (51–78) 92 (68–101) 70 (51–78) 128<sup>Δ</sup> (1248–1415) 199 (144–234)

K<sub>m</sub> value for (R)-ketamine metabolism was 1.3-fold higher than that for (S)-ketamine, leading to an approximately 27% decrease in the C<sub>int</sub> value for (R)-ketamine metabolism, which is similar to the data previously reported by Portmann et al., who examined ketamine metabolism using the same recombinant CYP2B6 system (Portmann et al., 2010). Of interest, the COS-1 cell–expressed CYP2B6<sup>f</sup> protein did not exhibit stereoselectivity in ketamine N-demethylation. This conflicting result is possibly the consequence of the Cyt b5–induced conformational changes on CYP2B6 protein. Although this effect on CYP2B6 has not been reported, Cyt b5 has been associated with conformational changes in CYP3A4 and CYP2C9 protein that can increase the collision between the substrate and the active-oxygen species at the active site of enzyme (Perret and Pompon, 1998; Locuson et al., 2007).

The present results are consistent with the finding of our preliminary clinical data, that is, a significant reduction in the plasma norketamine to ketamine concentration ratio in the CYP2B6<sup>f</sup> carriers (unpublished data). Although further follow-up work is required to examine the influence of this variant on ketamine metabolism in vivo, our in vitro findings imply that genotyping of CYP2B6<sup>f</sup> allele may be useful in estimating ketamine clearance rate and for predicting drug interactions that might be attributed to CYP2B6 and CYP3A4 and, thus, may help to guide dosing decision and establish a safer dosage regimen. Because ketamine is a high heparically cleared drug with low (~25%) oral bioavailability, this proposal would be more relevant when ketamine is given orally.

In conclusion, here, we demonstrate that the most common allelic variant of CYP2B6 gene, CYP2B6<sup>f</sup>, is associated with the decrease in both enzyme ketamine binding and N-demethylation activity. To the best of our knowledge, this is the first report showing the impact of
CYP2B6*6 and Ketamine Metabolism

Fig. 6. Effect of CYP2B6 and CYP3A4 inhibitors on norketamine formation from both low and high ketamine concentrations in HLMs with CYP2B6*1/*1 (n = 4), CYP2B6*1/*6 (n = 4), and CYP2B6*6/*6 (n = 3) genotypes. Low substrate concentrations were equivalent to the relative Km2 values; high substrate concentrations were equivalent to the relative Km1 values. MAB-3A, monoclonal antibody against CYP3A; MAB-2B6, monoclonal antibody against CYP2B6; TAO, troleandomycin. Results represent the mean ± S.D. **Significant inhibition P < 0.05; ***Significant gene-dose effect on inhibitory effects among three genotype groups, P < 0.01.

Acknowledgments

The authors thank Joel Colvill for assistance with HPLC assay; Dr. Daniel Barratt for help in genotyping assays; and Dr. Benjamin Lewis for help in carbon monoxide difference spectrum assay.

Authorship Contributions

Participated in research design: Li, Coller, Hutchinson, Klein, Zanger, Somogyi.

Conducted experiments: Li.

Contributed new reagents or analytic tools: Coller, Klein, Zanger, Stanley, Abell, Somogyi.

Performed data analysis: Li, Coller, Somogyi.

Wrote or contributed to the writing of this manuscript: Li, Coller, Hutchinson, Klein, Zanger, Stanley, Abell, Somogyi.

References


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