### INVOLVEMENT OF CYP2B6 IN N-DEMETHYLATION OF KETAMINE IN HUMAN LIVER MICROSONES

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**ABSTRACT:**

Ketamine is metabolized by cytochrome P450 (CYP) leading to production of pharmacologically active products and contributing to drug excretion. We identified the CYP enzymes involved in the N-demethylation of ketamine enantiomers using pooled human liver microsomes and microsomes from human B-lymphoblastoid cells that expressed CYP enzymes. The kinetic data in human liver microsomes for the (R)- and (S)-ketamine N-demethylation activities could be analyzed as two-enzyme systems. The \( K_m \) values were 31 and 496 \( \mu M \) for (R)-ketamine, and 24 and 444 \( \mu M \) for (S)-ketamine. Among the 12 cDNA-expressed CYP enzymes examined, CYP2B6, CYP2C9, and CYP3A4 showed high activities for the N-demethylation of both enantiomers at the substrate concentration of 1 \( \mu M \). CYP2B6 had the lowest \( K_m \) value for the N-demethylation of (R)- and (S)-ketamine (74 and 44 \( \mu M \), respectively). Also, the intrinsic clearance (\( Cl_{int} / V_{max} / K_m \)) of CYP2B6 for the N-demethylation of both enantiomers were 7 to 13 times higher than those of CYP2C9 and CYP3A4. Orphenadrine (CYP2B6 inhibitor, 500 \( \mu M \)) and sulfaphenazole (CYP2C9 inhibitor, 100 \( \mu M \)) inhibited the N-demethylase activities for both enantiomers (5 \( \mu M \)) in human liver microsomes by 60 to 70%, whereas cyclosporin A (CYP3A4 inhibitor, 100 \( \mu M \)) failed to inhibit these activities. In addition, the anti-CYP2B6 antibody inhibited these activities in human liver microsomes by 80%, whereas anti-CYP2C antibody and anti-CYP3A4 antibody failed to inhibit these activities. These results suggest that the high affinity/low capacity enzyme in human liver microsomes is mediated by CYP2B6, and the low affinity/high capacity enzyme is mediated by CYP2C9 and CYP3A4. CYP2B6 mainly mediates the N-demethylation of (R)- and (S)-ketamine in human liver microsomes at therapeutic concentrations (5 \( \mu M \)).

Ketamine \([R, S-2-(O-chlorophenyl)-2-(methylamino)cyclohexanone]\) is an analgesic and anesthetic agent which has been used in clinical studies as a racemate (White et al., 1982; Reich and Silvay, 1989). (S)-Ketamine is four times more potent as an analgesic than (R)-ketamine (Mathisen et al., 1995). N-Desmethylketamine (norketamine), which is the main metabolite of ketamine, may also contribute to the analgesic effects following ketamine administration (Shimoyama et al., 1999).

The in vivo and in vitro metabolisms of the racemic ketamine have been studied in humans and various animal species. Studies on human subjects given tritium-labeled ketamine have shown that 91% of the drug excretion. We identified the CYP enzymes involved in the N-demethylation of ketamine enantiomers using pooled human liver microsomes and microsomes from human B-lymphoblastoid cells that expressed CYP enzymes. The kinetic data in human liver microsomes for the (R)- and (S)-ketamine N-demethylation activities could be analyzed as two-enzyme systems. The \( K_m \) values were 31 and 496 \( \mu M \) for (R)-ketamine, and 24 and 444 \( \mu M \) for (S)-ketamine. Among the 12 cDNA-expressed CYP enzymes examined, CYP2B6, CYP2C9, and CYP3A4 showed high activities for the N-demethylation of both enantiomers at the substrate concentration of 1 \( \mu M \). CYP2B6 had the lowest \( K_m \) value for the N-demethylation of (R)- and (S)-ketamine (74 and 44 \( \mu M \), respectively). Also, the intrinsic clearance (\( Cl_{int} / V_{max} / K_m \)) of CYP2B6 for the N-demethylation of both enantiomers were 7 to 13 times higher than those of CYP2C9 and CYP3A4. Orphenadrine (CYP2B6 inhibitor, 500 \( \mu M \)) and sulfaphenazole (CYP2C9 inhibitor, 100 \( \mu M \)) inhibited the N-demethylase activities for both enantiomers (5 \( \mu M \)) in human liver microsomes by 60 to 70%, whereas cyclosporin A (CYP3A4 inhibitor, 100 \( \mu M \)) failed to inhibit these activities. In addition, the anti-CYP2B6 antibody inhibited these activities in human liver microsomes by 80%, whereas anti-CYP2C antibody and anti-CYP3A4 antibody failed to inhibit these activities. These results suggest that the high affinity/low capacity enzyme in human liver microsomes is mediated by CYP2B6, and the low affinity/high capacity enzyme is mediated by CYP2C9 and CYP3A4. CYP2B6 mainly mediates the N-demethylation of (R)- and (S)-ketamine in human liver microsomes at therapeutic concentrations (5 \( \mu M \)).

1 Abbreviations used are: CYP, cytochrome P450; \( Cl_{int} \), intrinsic clearance; G-6-P, glucose-6-phosphate; NADP, nicotinamide-adenine dinucleotide phosphate; HPLC, high performance liquid chromatography.

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**Chemicals.** The enantiomers of ketamine and norketamine were kindly supplied by Parke-Davis (Ann Arbor, MI). Orphenadrine, sulfaphenazole, and cyclosporin A were purchased from Sigma Chemical Co. (St. Louis, MO). \( \beta \)-NADP\(^{+} \), glucose-6-phosphate (G-6-P), MgCl\(_2\)-6H\(_2\)O, and G-6-P dehydrogenase were purchased from Wako Pure Chemical Co. (Osaka, Japan). Microsomes from human B-lymphoblastoid cell lines expressing different human CYP enzymes (CYP1A1, 1A2, 1B1, 2A6, 2B6, 2C8, 2C9, 2C19, 2D6, 2E1, 3A4, 4A11; 11.5–11.5 pmol of CYP/\( mg \) of protein) and control microsomes from human B-lymphoblastoid cell lines containing the expression vector without \( cDNA \) of CYP were purchased from GENTEST Co. (Woburn, MA). Pooled human liver microsomes (450 pmol of CYP/\( mg \) of protein), which is a mixture of microsomes prepared from 10 individual donors, monoclonal antibodies raised against human CYP2B6 and CYP3A4, and a polyclonal antibody against human CYP2C were also purchased from GENTEST Co. The immunochromically determined CYP contents of the pooled human liver microsomes were provided in the data sheet supplied by the manufacturer as follows: (pmol of CYP/\( mg \) of protein): CYP1A1, 50; 2A6, 60; 2B6, 26; 2C9, 55; 2D6, 11; 2E1, 53; 3A4, 127; and 3A5, 3.

**Typical Assay Conditions.** The incubation medium contained 1 \( mg/ml \) cDNA-expressed CYP enzymes or 0.4 \( mg/ml \) human liver microsomes, a
NADPH-generating system (1.3 mM NADP+, 3.3 mM G-6-P, 0.4 U/ml G-6-P dehydrogenase, 3.3 mM MgCl2), various concentrations of substrate [(R)- or (S)-ketamine], and 50 mM Tris-HCl buffer (pH 7.4) or 50 mM potassium-phosphate buffer (pH 7.4), in a final volume of 0.5 ml. After preincubation at 37°C for 3 min, the reaction was initiated by the addition of the cDNA-expressed CYP enzymes or NADP+ (for assay with human liver microsomes). The reaction was stopped by the addition of 0.25 ml of 1 M carbonate buffer (pH 10.5), and the metabolites were extracted into 5 ml of cyclohexane. The mixture was centrifuged at 1600 g for 10 min, and then the organic layer was evaporated to dryness. The residue was dissolved in 100 µl of a mobile phase of HPLC, and 50 µl was subjected to HPLC as described below. The N-demethylation activities of ketamine were evaluated from the formation rate of norketamine. The (R)- or (S)-norketamine formations were linear throughout the 30-min incubation period in expressed P450s and the 10-min incubation period in human liver microsomes.

**Kinetics in Human Liver Microsomes**. The (R)- and (S)-ketamine N-demethylation activities in the pooled human liver microsomes were determined at substrate concentrations ranging from 10 to 2000 µM. Incubations were carried out for 10 min at 37°C. Control experiments using the incubation medium without NADP+ were run in parallel. Nonenzymatic formation of norketamine was not observed. The kinetic parameters were estimated as described below.

**Assay with cDNA-Expressed CYP Enzymes**. To examine the roles of individual CYP enzymes involved in the ketamine N-demethylation, each of the 12 cDNA-expressed CYP enzymes and the control microsomes were incubated with 1 mM (R)- or (S)-ketamine for 30 min at 37°C. Kinetic studies were performed for CYP2B6, CYP2C9, and CYP3A4. The (R)- and (S)-ketamine N-demethylation activities for CYP2B6 were determined at substrate concentrations ranging from 10 to 1000 µM, and for CYP2C9 and CYP3A4 ranging from 62.5 to 2000 µM. Incubations were carried out for 10 min at 37°C. The kinetic parameters were estimated as described below.

**Inhibition Studies in Human Liver Microsomes**. Orphenadrine, sulfaphenazole and cyclosporin A were added to the human liver microsomal incubations at a final concentration of 1 to 500 µM. Incubation medium containing orphenadrine (20, 100, and 500 µM) was preincubated with all the components of the reaction except for (R)- or (S)-ketamine for 15 min at 37°C. The reaction was initiated by the addition of the substrate at a final concentration of 5 µM. Sulfaphenazole (1, 10, and 100 µM) and cyclosporin A (1, 10, and 100 µM) were added to the incubation medium containing 5 µM (R)- or (S)-ketamine. After preincubation at 37°C for 3 min, the reaction was initiated by the addition of NADP+ and carried out for 10 min.

Various amounts of the antibodies against CYP2B6, CYP2C, and CYP3A4 were added to the incubation medium containing 5 µM (R)- or (S)-ketamine, and the medium was preincubated for 30 min at 25°C. Following the preincubation at 37°C for 3 min to equilibrate the temperature, the reaction was initiated by the addition of NADP+ and carried out for 10 min at 37°C.

**HPLC Conditions**. (R)- and (S)-norketamine were separated and measured by HPLC (Yanagihara et al., 2000). The HPLC system consisted of a LC-6A pump, a CTO-6A column oven, an SPD-6A UV detector and a C-R1B data-acquisition system. The mobile phase consisted of 88% n-hexane and 12% 2-propanol (98.2, v/v). The flow rate of the mobile phase was 0.8 ml/min. The detection wavelength was 215 nm. No interfering peak for (R)- and (S)-norketamine on chromatograms were observed when the incubation medium was analyzed. The retention times of (R)- and (S)-ketamine were 25 and 27 min, respectively.

**Data Analysis**. The kinetic parameters (Km, Vmax) were estimated by a nonlinear least-squares regression analysis program, MULTI (Yamaoka et al., 1981).

**Results and Discussion**. (R)- and (S)-ketamine N-demethylation in human liver microsomes exhibited biphasic kinetics, evidenced by nonlinear Eadie-Hofstee plots (data not shown), indicating the catalytic participation of more than one enzyme. The data were best fit by a two-enzyme model (Table 1). A high affinity/low capacity enzyme exhibited Km1 values of 31.4 and 24.1 µM, and Vmax1 values of 3.3 and 2.0 pmol/min/pmol of CYP for (R)- and (S)-ketamine, respectively. A low affinity/high capacity enzyme exhibited Km2 values of 495.7 and 443.9 µM and Vmax2 values of 10.2 and 10.2 pmol/min/pmol of CYP for (R)- and (S)-ketamine, respectively. The Clint1 (Vmax1/Km1) for the N-demethylation of (R)- and (S)-ketamine were 107.0 ml/min/pmol of CYP and 132.5 ml/min/pmol of CYP, respectively. Clint values were 5.3 to 5.8 times higher than the Clint2 (Vmax2/Km2), suggesting that the N-demethylation is predominantly catalyzed by one enzyme (the high affinity/low capacity enzyme) at therapeutic concentrations (5 µM). Kharasch and Labroo (1992) have reported that N-demethylation of ketamine enantiomers exhibited biphasic kinetics, being best fit by a two-enzyme model in three individual human liver microsomal preparations, and the rate of the N-demethylation of (S)-ketamine was 20% greater than that of (R)-ketamine. However, there were no stereoselective differences in the N-demethylation activities of ketamine in human liver microsomes in our study.

**TABLE 1**

<table>
<thead>
<tr>
<th></th>
<th>Km1</th>
<th>Vmax1</th>
<th>Clint1</th>
<th>Km2</th>
<th>Vmax2</th>
<th>Clint2</th>
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<tr>
<td>(R)-Ketamine</td>
<td>31.4 ± 6.2</td>
<td>3.3 ± 0.5</td>
<td>107.0 ± 5.3</td>
<td>495.7 ± 90.4</td>
<td>10.2 ± 0.6</td>
<td>20.9 ± 3.3</td>
</tr>
<tr>
<td>(S)-Ketamine</td>
<td>24.1 ± 4.8</td>
<td>3.2 ± 0.5</td>
<td>132.5 ± 7.7</td>
<td>443.9 ± 77.7</td>
<td>10.2 ± 0.3</td>
<td>23.5 ± 4.3</td>
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</tbody>
</table>

**FIG. 1.** Ketamine N-demethylase activities in microsomes from human B-lymphoblastoid cell lines expressing CYP enzymes.

(R)-Ketamine or (S)-ketamine (1 mM) was incubated with the microsomes from human B-lymphoblastoid cell lines expressing different human CYP enzymes for 30 min at 37°C. Each value represents the mean ± S.D. of three independent experiments. □, (R)-ketamine; □, (S)-ketamine.
Table 2

Kinetic parameters for the N-demethylation of (R)- and (S)-ketamine by microsomes from human B-lymphoblastoid cell lines expressing CYP2B6, CYP2C9, and CYP3A4

Each value represents the mean ± S.D. of three independent experiments.

<table>
<thead>
<tr>
<th></th>
<th>(R)-Ketamine</th>
<th></th>
<th>(S)-Ketamine</th>
<th></th>
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<tbody>
<tr>
<td></td>
<td>$k_m$</td>
<td>$V_{max}$</td>
<td>$CL_{int}$</td>
<td>$k_m$</td>
</tr>
<tr>
<td>CYP2B6</td>
<td>74.1 ± 34.2</td>
<td>33.3 ± 5.0</td>
<td>526 ± 264</td>
<td>44.4 ± 9.6</td>
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<tr>
<td>CYP2C9</td>
<td>521.4 ± 122.7</td>
<td>28.6 ± 1.7</td>
<td>57 ± 12</td>
<td>756.2 ± 85.8</td>
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<tr>
<td>CYP3A4</td>
<td>711.3 ± 250.2</td>
<td>51.6 ± 8.3</td>
<td>76 ± 16</td>
<td>399.0 ± 48.3</td>
</tr>
</tbody>
</table>

Fig. 2. Effects of chemical inhibitors (A) and anti-CYP antibodies (B) on the N-demethylation of (R)- and (S)-ketamine in human liver microsomes.

(R)-Ketamine or (S)-ketamine (5 μM) was incubated with the human liver microsomes in the presence of chemical inhibitors or anti-CYP antibodies for 10 min at 37°C. Activities are expressed as a percentage of control (absence of chemical CYP inhibitor or anti-CYP antibodies). Each value represents the mean ± S.D. of three independent experiments. ●, (R)-ketamine; ○, (S)-ketamine.

Microsomes from human B-lymphoblastoid cell lines expressing each of the 12 CYP enzymes were investigated to identify the capacity of individual CYP enzymes to catalyze N-demethylation of (R)- and (S)-ketamine (Fig. 1). Among the 12 CYP enzymes, CYP2B6, CYP2C9, and CYP3A4 showed high activities (9.3–27.6 pmol/min/pmol of CYP) for the N-demethylation of both enantiomers at substrate concentrations of 1 mM. The other CYP enzymes exhibited low activities (less than 3.0 pmol/min/pmol of CYP) for the N-demethylation of both enantiomers. Kinetic parameters for the N-demethylation of (R)- and (S)-ketamine were estimated for CYP2B6, CYP2C9, and CYP3A4 (Table 2). The $k_m$ values of CYP2B6 for the N-demethylation of (R)- and (S)-ketamine (74.1 and 44.4 μM, respectively) were 7 to 17 times lower than those of CYP2C9 and CYP3A4 (399.0–756.2 μM). The $k_m$ values of CYP2B6 were similar to those for the high affinity/low capacity enzyme in the human liver microsomes, and those of CYP2C9 and CYP3A4 were similar to those for the low affinity/high capacity enzyme. The $V_{max}$ values of the three enzymes were equal. There were no stereoselective differences in the kinetic parameters of the three enzymes. The $K_m$ values of CYP2B6, CYP2C9, and CYP3A4 were similar to those for the low affinity/low capacity enzyme in the human liver microsomes, and those of CYP2C9 and CYP3A4 were similar to those for the low affinity/high capacity enzyme.

The effects of chemical inhibitors and anti-CYP antibodies on N-demethylation of (R)- and (S)-ketamine were investigated in human liver microsomes using substrate concentration of 5 μM (Fig. 2). Orphenadrine, an inhibitor of CYP2B6 at 500 μM, inhibited (R)- and (S)-ketamine N-demethylase activities by 67 and 64%, respectively. Sulfaphenazole, an inhibitor of CYP2C9 at 100 μM, also inhibited the activities by 62 and 57%, respectively. Cyclosporin A, an inhibitor of CYP3A4 at 100 μM, did not inhibit the N-demethylation of (R)- or (S)-ketamine. The antibodies against CYP2B6 inhibited in the (R)- and (S)-ketamine N-demethylase activities by about 80%, whereas the antibodies against CYP2C2 and CYP3A4 failed to inhibit these activities. Newton et al. (1995) reported that sulfaphenazole was a selective inhibitor of CYP2C9, but they did not study the effect of sulfaphenazole on the CYP2B6-mediated reactions. We found that sulfaphenazole (100 μM) produced approximately 60% decrease in the ketamine N-demethylase activities of recombinant CYP2B6. Our data suggest that sulfaphenazole may be an inhibitor of CYP2B6.

Drug interactions are clinically important. However, there are few reports about drug interactions with ketamine in humans. Premedication with diazepam, a substrate of CYP2C19 and CYP3A4, or seco- barbital, an inhibitor of CYP2B, increased plasma half-lives of ketamine (10 mg/kg, i.m.) in humans (Lo and Cumming, 1975). The metabolism of bupivacaine was inhibited by ketamine (10 mg/kg, i.p.) in mice (Gantenbein et al., 1997). Ketamine (100 mg/kg, i.p.) decreased the clearances of flecainide, a substrate of CYP2D1, and ethosuximide, a substrate of CYP3A, by 13 to 18% in rats (Loch et al., 1995). Pretreatment of rat and rabbit with phenobarbital, an inducer of the CYP2B subfamily, causes a marked increase in the rate of ketamine metabolism by hepatic tissue in vitro (Woolf and Adams, 1995). In this study, we showed that a low concentration of ketamine (5 μM) was metabolized by CYP2B6 in human liver microsomes. Ifosphamide and cyclophosphamide, anticancer agents, are activated by CYP2B6 (Chang et al., 1993). Propofol, an anesthetic drug, has been demonstrated to exhibit a concentration-dependent inhibitory effect on CYP2B1 (Chen et al., 1995). Phenobarbital, dexamethasone,
and rifampin induce CYP2B6 (Chang et al., 1997). These substrates and inducers may affect the N-demethylation of ketamine.

In conclusion, the results of the present study using human liver microsomes and human cDNA-expressed CYP enzymes indicate that CYP2B6 is the main enzyme mediating the N-demethylation of (R)- and (S)-ketamine in human liver microsomes at therapeutic concentrations.

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References