CONTRIBUTION OF CYP3A4, CYP2B6, AND CYP2C9 ISOFORMS TO N-DEMETHYLATION OF KETAMINE IN HUMAN LIVER MICROSOMES

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ABSTRACT:
Ketamine is a widely used drug for its anesthetic and analgesic properties; it is also considered as a drug of abuse, as many cases of ketamine illegal consumption were reported. Ketamine is N-demethylated by liver microsomal cytochrome P450 into norketamine. The identification of the enzymes responsible for ketamine metabolism is of great importance in clinical practice. In the present study, we investigated the metabolism of ketamine in human liver microsomes at clinically relevant concentrations. Liver to plasma concentration ratio of ketamine was taken into consideration. Pooled human liver microsomes and human lymphoblast-expressed P450 isoforms were used. N-demethylation of ketamine was correlated with nifedipine oxidase activity (CYP3A4-specific marker reaction), and it was also correlated with S-mephenytoin N-demethylase activity (CYP2B6-specific marker reaction). Orphenadrine, a specific inhibitor to CYP2B6, and ketoconazole, a specific inhibitor to CYP3A4, inhibited the N-demethylation of ketamine in human liver microsomes. In human lymphoblast-expressed P450, the activities of CYP2B6 were higher than those of CYP3A4 and CYP2C9 at three concentrations of ketamine, 0.005, 0.05, and 0.5 mM. When these results were extrapolated using the average relative content of these P450 isoforms in human liver, CYP3A4 was the major enzyme involved in ketamine N-demethylation. The present study demonstrates that CYP3A4 is the principal enzyme responsible for ketamine N-demethylation in human liver microsomes and that CYP2B6 and CYP2C9 have a minor contribution to ketamine N-demethylation at therapeutic concentrations of the drug.

FIG. 1. The N-demethylation pathway of ketamine in hepatic cytochrome P450. *

Ketamine Norketamine

N-demethylation

H3C-\text{NH}O

\text{Cl}

\text{Cl}

H2N

+ chiral carbon.

Ketamine is a N-methyl-D-aspartate receptor antagonist used in clinical practice since 1970 for its anesthetic, sedative, and analgesic properties. It is frequently used for induction of anesthesia in short term surgical operations, in maintaining anesthesia with other anesthetic agents, and as a postoperative pain relief agent in intensive care units (Nimmo and Clements, 1981; White et al., 1982). In recent years, there is increasing evidence of ketamine abuse, many cases of illegal ketamine consumption, and dependence was reported (Jansen et al., 1994). In light of these studies, and in the view of the growing interest of ketamine both as a therapeutic agent and as a drug of abuse, the knowledge of the identity and the contribution of P450 enzymes to N-demethylation of ketamine in humans, at clinically relevant concentrations, is highly desired.

The present study was undertaken to identify the P450 isoforms responsible for the N-demethylation of ketamine, using a pool of human liver microsomes obtained from 20 donors. Specific chemical inhibitors and human lymphoblast-expressed P450 were used. The study was conducted at clinically relevant concentrations of ketamine, taking into consideration that liver to plasma ratio is about 3 (Moore et al., 1997). The contribution of the identified P450 isoforms to the
bioransformation of ketamine into norketamine at analgesic, anesthetic, and toxic plasma levels in humans was also examined by extrapolating the results obtained using the relative abundance of P450 isoforms available in the literature.

Materials and Methods

**Chemicals and Microsomal Preparations.** Ketamine, NK, and nortrilidine (internal standard) were generously supplied by Pfizer Laboratories (Paris, France). Acetonitrile, dichloromethane, and ethyl acetate (Uvasol grade), potassium dihydrogen phosphate, and boric acid were purchased from Elvetec (Lyon, France). Chemical inhibitors to cytochromes P450 and NADPH were purchased from Sigma-Aldrich (St. Quentin, France). Human liver microsomes from seven donors and pooled microsomal preparations prepared from 20 livers were obtained from Biopredic International (Rennes, France). Human lymphoblast-expressed CYP3A4, CYP2C9, and CYP2B6 were purchased from Interchim (Paris, France).

**Enzyme Incubation Conditions.** The typical incubation mixture consisted of 0.5 mg/ml human liver microsomes, 100 mM potassium phosphate buffer (pH 7.4), and substrate in a final volume of 0.5 ml incubated in polypolyene tubes, at 37°C. After preincubation for 3 min, the reactions were initiated by the addition of 1 mM NADPH. At the end of incubation time, 1 ml of cold 0.1 M NaOH was added to stop the enzymatic activity. All incubations were run in duplicates.

**In Vitro Half-Life Determination.** The in vitro half-life of ketamine was determined in a pool of human liver microsomes (20 donors). Ketamine at a concentration of 0.05 mM was incubated with 0.5 mg/ml microsomes under the same conditions as previously mentioned. Enzymatic reactions were terminated at T0 and at the following time intervals: 5, 10, 15, 20, 30, 60, 120, and 180 min. The half-life was determined based on substrate depletion and metabolite formation (see data analysis section).

**Kinetic Study.** The rate of norketamine formation was determined in a pool of human liver microsomes (20 donors) at microsomal concentrations of 0.5 mg/ml and at ketamine concentrations ranging from 0.0025 to 4 mM. The mixtures were incubated for 10 min under the same conditions as previously mentioned.

**Correlation Study.** Ketamine (0.05 mM) was incubated with human liver microsomes (0.5 mg/ml) obtained from seven donors. The livers showing remarkable variations in their cytochrome P450 activities were chosen for this study. Incubations were done for 60 min under the same conditions as previously mentioned.

**Inhibition Study.** The effect of specific chemical inhibitors to CYP3A4, CYP2B6, CYP2C9, CYP2D6, CYP1A2, CYP2A6, and CYP2C19 on ketamine N-demethylation activity was studied in a pool of human liver microsomes (20 donors). Two concentrations of the following inhibitors were used, 2 and 10 μM ketoconazole (CYP3A), 100 and 500 μM orphenadrine (CYP2B6), 10 and 50 μM sulfaphenazole (CYP2C9), 2 and 10 μM quinidine (CYP2D6), 100 and 500 μM phenacetin (CYP1A2), 100 and 500 μM coumarin (CYP2A6), and 1 and 10 μM omeprazole (CYP2C19). The inhibitors were dissolved in methanol, and then 20 μl of each solution was transferred to the incubation mixture. Twenty microliters of methanol were transferred to inhibitor-free control samples to cancel any possible effect of methanol on enzyme activity. All inhibitors were preincubated with ketamine (0.05 mM) and microsomes (0.5 mg/ml) in the phosphate buffer for 3 min, then the reaction was started with NADPH (1 mM) for 60 min, except for orphenadrine (mechanism based inhibitor), in which the mixture cofactor-buffer-microsome was incubated with the inhibitor for 15 min, then the reaction was initiated with ketamine and stopped after 60 min.

**Assay with Human Lymphoblast-Expressed P450 Isoforms.** Human lymphoblast-expressed CYP3A4, CYP2B6, and CYP2C9 were used. These recombinant P450 isoforms were incubated in quadruplicates at three substrate concentrations, 0.5, 0.05, and 0.005 mM. The incubation conditions recommended by the manufacturer were modified to obtain linear metabolite formation. Concerning CYP3A4 and CYP2C9, a concentration of 40 pmol/ml was used for all substrate levels. Meanwhile for CYP2B6, a concentration of 3, 40, and 80 pmol/ml for 0.005, 0.05, and 0.5 mM of ketamine were used, respectively. According to the manufacturer’s recommendations, recombinant P450 isoforms were thawed rapidly in a 37°C water bath, and then they were kept on ice until use. The enzymatic reaction was initiated by adding ice-cold microsomes to prewarmed buffer-substrate-cofactor mixture and incubated for 60 min, then the reaction was stopped and the samples were treated as previously described.

**HPLC Conditions.** Ketamine and norketamine were analyzed according to the chromatographic method described by Bolze and Boulieu (1998). The chromatographic system consisted of Hewlett Packard 1050 series with a computer HP Vectra 840/33M using HPCham software and an HP DeskJet 510 (Hewlett Packard, Palo Alto, CA). The column used was a reversed-phase silica gel end-capped Purospher RP-18 (5 μm) 125 × 4 mm purchased from Merck (Darmstadt, Germany). Briefly, 0.5 ml of each sample was alkalized with boric acid (pH 13) and extracted twice with a mixture of dichloromethane: ethyl acetate (80:20, v/v) followed by a back-extraction with 2 M HCl. After evaporation of the acid layer and reconstitution of the residue with mobile phase, 60 μl were injected into the HPLC column.

**Data Analysis.** Statistical analysis was done with Instat software (GraphPad Software, San Diego, CA). The nonparametric Spearman rank correlation test was used for correlation study between ketamine N-demethylase activity (as measured by NK formation rate) and various P450 isoform activities. Two-tailed p value <0.05 was considered statistically significant. The enzyme kinetic parameters (Km and Vmax) were initially evaluated by graphical examination of Eadie-Hofstee plot. These values were taken as initial parameters for the estimation of the Michaelis-Menten constants by nonlinear regression curve fitting using the GraphPad Inplot software (GraphPad Software).

The in vitro half-life of total ketamine disappearance was determined from the semilogarithmic plot of the assayed ketamine concentration versus time. That of the N-demethylation pathway was determined from the semilogarithmic plot of the concentration of ketamine not bioransformed into NK versus time. The concentration of ketamine not bioransformed into NK was calculated by subtracting NK concentration obtained at different time intervals from the initial concentration of ketamine in the incubation medium. The in vitro intrinsic clearance (Cl) was estimated by the following equation: Cl (in vitro) = V / (V/Km), where V is the incubation volume (Chenery et al., 1987). The Cl of the N-demethylation pathway was compared with that of overall Cl in human liver microsomes, and the contribution of this pathway was estimated.

To determine the contribution of CYP3A4, CYP2B6, and CYP2C9 in the N-demethylation of ketamine in human liver microsomes, the results were extrapolated by multiplying the rate of norketamine formation in recombinant P450 isoforms (expressed as picomoles per minute per picromoles P450) by the relative abundance of these isoforms in human liver microsomes based on pooled data obtained from the literature.

**Results**

**Enzyme Kinetics.** The enzyme kinetics of ketamine N-demethylation was biphasic as shown in Eadie-Hofstee plot (Fig. 2). The data were fit in a double rectangular hyperbolic plot. This indicates that at least two enzyme systems are responsible for the N-demethylation pathway of ketamine. The Vmax value was 5 pmol/min/pmol P450, Km value was 47.5 μM, and the intrinsic clearance (Vmax/Km) was 106 × 10⁻⁶ ml/min/pmol P450 for the high affinity/low capacity enzyme. For the low affinity/high capacity enzyme, Vmax, Km, and Vmax/Km were 14 pmol/min/pmol P450, 2260 μM, and 6.2 × 10⁻⁶ ml/min/pmol P450, respectively. These values were close to those reported by Yanagihara et al. (2001) and Kharasch and Labroo (1992), except that Km of the low affinity/high capacity enzyme was higher in our study than reported in literature. This might be due to the fact that the previous studies were conducted on ketamine enantiomers, whereas in our experiment we used the racemate.

**In Vitro Half-Life.** The in vitro half-lives of total ketamine metabolism and that of ketamine N-demethylation were determined in a pool of human liver microsomes (data not shown), then the corresponding intrinsic clearances were calculated. The in vitro total intrinsic clearance of ketamine in human liver microsomes was 23.3 × 10⁻⁶ ml/min/pmol P450 and that of the N-demethylation pathway was...
Correlation Analysis. Ketamine N-demethylation activity was significantly correlated with nifedipine oxidase activity and S-mephenytoin N-demethylation activity in seven human liver microsomal preparations. No significant correlation was found between the other principal P450 isoform activities and ketamine N-demethylation (Table 1). The liver preparations used in this study showed a good variability in the activities of the various P450 isoforms toward specific marker reactions as determined by the manufacturer. The incubation concentration of ketamine used was 0.05 mM, which corresponds to therapeutic plasma levels of about 4000 ng/ml observed in patients anesthetized by ketamine, taking into consideration that the liver to blood concentration ratio of ketamine is equal to 2.72 (Moore et al., 1997) and that the blood to plasma concentration ratio is equal to 1 ( Hijazi et al., 2001). The choice of this concentration of ketamine was also based upon the enzyme kinetic results. Lower concentrations of ketamine in the incubation mixture could overestimate the contribution of the high affinity/low capacity enzyme to ketamine metabolism.

Inhibition Study. The effects of seven chemical inhibitors of the principal P450 isoforms were studied at therapeutic concentration of ketamine (0.05 mM) in a pool of 20 human liver microsomes. The choice of inhibitors and their concentrations was based on literature data (Reidy et al., 1989; Halpert et al., 1994; Rodrigues, 1994; Baldwin et al., 1995; Bourrie et al., 1996). The low concentration used for each inhibitor might inhibit the specificity of the P450 isoform-specific reaction with a specific marker substrate. The high concentration used for each inhibitor might inhibit more than 80% of the P450 isoform-specific activity, but the inhibitor starts to be less selective (Rodrigues, 1994). As shown in Fig. 3, ketoconazole (2 μM), a potent and selective CYP3A inhibitor, inhibited N-demethylation of ketamine by about 40%. At 10 μM ketoconazole, about 65% of N-demethylation activity was inhibited. Orphenadrine, an inhibitor of CYP2B6, inhibited norketamine formation by about 20% and by about 60% at inhibitor concentration of 100 and 500 μM, respectively. Minor inhibition was observed with both phenacetin (CYP1A2 inhibitor) and coumarin (CYP2A6 inhibitor) with little effect of the concentration of inhibitor used. Omeprazole at 10 μM (CYP2C19 inhibitor) inhibited by less than 15% the N-demethylation pathway, but this may be due to loss of selectivity and the possibility of CYP3A inhibition at high concentration of omeprazole (Ko et al., 1997).

Study in Human Lymphoblast-Expressed P450 Isoforms. As shown in Fig. 4A, the rate of NK formation expressed as picomoles per minute per picomoles P450 was higher with CYP2B6 than with CYP3A4 and with CYP2C9 at the three concentrations of ketamine examined. These results were extrapolated to human liver microsomes by multiplying the turn over number obtained in recombinant P450 by the relative abundance of these isoforms in human liver (Iwatsubo et al., 1998; Becquemont et al., 1998). The average content of these isoforms was obtained by pooling data from literature (Guengerich and Turvy, 1991; Shimada et al., 1994; Code et al., 1997; Becquemont et al., 1998; Ekins et al., 1998; Lasker et al., 1998; Shimada et al., 1999). In 184 individuals, the average content of CYP3A4 was 146 pmol/mg of human liver microsomes and that of CYP2C9 was 89 pmol/mg of human liver microsomes (considering that CYP2C9 constitute one-half of the total CYP2C content). In 120 individuals, the average content of CYP2B6 was 4.8 pmol/mg of human liver microsomes. The estimated values of ketamine N-demethylation (Fig. 4B)
indicated that CYP3A4 is the major enzyme involved in the biotransformation of ketamine into norketamine in human liver microsomes and that CYP2B6 and CYP2C9 have minor contributions to this pathway. These results also showed that at lower concentrations of substrate the relative contribution of CYP2B6 increases although that of CYP2C9 and CYP3A4 decreases. At 0.005 mM ketamine that corresponds to an analgesic plasma level, the contribution of CYP2B6 to ketamine N-demethylation is about one-half that of CYP3A4, and the contribution of CYP2C9 is about one-tenth that of CYP3A4 (Fig. 4B).

**Discussion**

The present study suggested that CYP3A4 isoform is the major enzyme responsible for N-demethylation of ketamine in human liver microsomes. Our results confirmed that NK is the major metabolite in human liver microsomes as determined by the ratio of the intrinsic clearance of ketamine N-demethylation pathway to that of the overall ketamine metabolism in human liver microsomes.

The results of the present paper support the following observations.

Significant correlations between ketamine N-demethylation activity and CYP3A4- and CYP2B6-specific activities in microsomes were observed. Ketoconazole inhibited ketamine N-demethylation by about 40% and by about 65% at inhibitor concentration of 2 μM and 10 μM, respectively, whereas orphenadrine inhibits ketamine N-demethylation by about 20% and by about 60% at inhibitor concentration of 100 and 500 μM, respectively, in human liver microsomes.

The use of lymphoblast-expressed CYP2B6, CYP2C9, and CYP3A4 demonstrated that CYP2B6 showed higher N-demethylation activity expressed as picomoles per minute per picomoles P450 than that of CYP3A4 and CYP2C9 at three concentrations of substrate tested. These results were extrapolated using the relative abundance of these P450 isoforms in human liver obtained from pooling the data available in the literature. An extensive interindividual variability of CYP2B6 expression in human livers was recently reported, and it could be due to genetic polymorphism (Lang et al., 2001).

These results showed that CYP3A4 is expected to be the major enzyme responsible for ketamine N-demethylation in humans at analgesic, anesthetic, as well as toxic plasma levels of the drug, and
CYP2B6 and CYP2C9 have a minor role in this metabolic pathway of ketamine. However, the contribution of CYP2B6 increases as the concentration of ketamine decreases. At 0.5 mM ketamine (toxic levels), CYP2B6 contribution to ketamine N-demethylation was about one-tenth that of CYP3A4, whereas at 0.005 mM (analgesic levels) CYP2B6 showed a relative contribution of about one-half that of CYP3A4. The results obtained in this paper concerning the identity of P450 isoforms involved in ketamine N-demethylation, were similar to those recently reported by Yanagihara et al. (2001), who reported that CYP2B6 is the high affinity/low capacity enzyme, whereas CYP3A4 and CYP2C9 were considered as the low affinity/high capacity enzymes, involved in the N-demethylation of ketamine in human liver microsomes. However, in Yanagihara et al. study, CYP2B6 was considered as the major isoform involved in ketamine N-demethylation. Moreover, anti-CYP2C and anti-CYP3A4 antibodies failed to inhibit the N-demethylation of ketamine in human liver microsomes; meanwhile, about 80% of norketamine formation was inhibited by anti-CYP2B6 antibodies. In fact, the concentration of ketamine used in the inhibition experiment of Yanagihara et al. was 0.005 mM, whereas in our experiment, ketamine concentration was 0.05 mM. Since CYP2B6 is the high affinity/low capacity enzyme, its relative contribution to ketamine metabolism increases at lower concentration of the substrate. This reason, in addition to the abundance consideration could explain the differences obtained between the conclusions of the two studies.

In the inhibition experiment in human liver microsomes, the results are not in total agreement with those in human lymphoblast-expressed P450. The participation of CYP2B6 to ketamine metabolism was equally important to that of CYP3A4, and no participation of CYP2C9 was observed at 0.05 mM of ketamine. These discrepancies between the results obtained from human liver microsomes and recombinant P450 systems may be due to the difference in activities per unit enzyme that may result from the difference between the levels of cytochrome oxidoreductase and accessory proteins in the two systems (Yamazaki et al., 1996; Venkatakrishnan et al., 2000). As long as validated scaling factors are needed to bridge the gap between recombinant P450 expression systems and human liver microsomes, experiments done in human liver microsomes remain the gold standard in determining the enzymes involved in the metabolism of drugs.

The involvement of CYP3A4, CYP2B6, and CYP2C9 in ketamine metabolism could be the basis of many potential pharmacokinetic drug interactions. Well known inhibitors of CYP3A4 such as azole antifungals, macrolide antibacterials, human immunodeficiency virus protease inhibitors, and cyclosporin; inhibitors to CYP2C9 such as statins anticholesterolemics; as well as inhibitors of CYP2B6 such as orphenadrine might increase plasma levels of ketamine. Meanwhile, inducers to CYP3A4, CYP2C9, and CYP2B6 such as barbiturates and rifampicin could enhance ketamine metabolism. In animals, cyclophosphamide, an anticancer agent and a CYP2B6 substrate, increased the duration of ketamine anesthesia in mice (Rojavin et al., 1996). Medetomidine, a CYP3A4 and CYP2C9 inhibitor, inhibited N-demethylation of ketamine in vitro experiments (Kharasch et al., 1992). In humans, patients with a history of chronic barbiturate use were associated with decreased steady-state plasma concentrations of ketamine (Koppel et al., 1990). This evidence is in agreement with our in vitro results since barbiturates are well known inducers to CYP3A4, CYP2C9, and CYP2B6. Few reports are available about pharmacokinetic drug interactions of ketamine, so clinical investigations in studying drug interactions of ketamine with drugs that are known to be substrates, inducers, or inhibitors to CYP3A4, CYP2C9, and CYP2B6 are urgently demanded. Interindividual variability and genetic polymorphism in CYP2B6 and CYP2C9 expression should be taken into consideration.

In conclusion, the results reported in this paper indicate that CYP3A4 is the major enzyme responsible for the biotransformation of ketamine into norketamine in human liver microsomes at therapeutic concentrations. These results also suggested that CYP2C9 and CYP2B6 have a minor role in ketamine N-demethylation in vivo.

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References


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