CYTOCHROME P-450 3A4 AND 2C8 ARE INVOLVED IN ZOPICLONE METABOLISM

LAURENT BECQUEMONT, SAID MOUAJJAH, OLIVIER ESCAFFRE, PHILIPPE BEAUNE, CHRISTIAN FUNCK-BRENTANO, AND PATRICE JAILLON

Clinical Pharmacology Unit (L.B., S.M., O.E., C.F.-B., P.J.), Saint Antoine University Hospital, School of Medicine Paris 6, France; and Institut National de la Santé et de la Recherche Médicale U 490 (P.B.), Saint-Pères University, School of Medicine Paris 5, France

(Received December 31, 1998; accepted May 7, 1999)

This paper is available online at http://www.dmd.org

ABSTRACT:

Zopiclone is a γ-aminobutyrinic acid receptor agonist that is widely prescribed for its hypnotic properties (Noble et al., 1998) in insomniac patients. As benzodiazepines agents, the duration of its pharmacological effect and the occurrence of side effects, such as morning hypnotic residual effects (Allain et al., 1991), are mainly dependent on its hepatic clearance.

In humans, zopiclone elimination is mainly dependent on its hepatic metabolism because only 5% of the drug is excreted unchanged in the urine (Noble et al., 1998). Indeed, zopiclone is extensively metabolized by the human liver into two major metabolites (Fig. 1): N-desmethyl-zopiclone (ND-Z) and N-oxide-zopiclone (NO-Z) with the following \( K_m \) and \( V_m \) of 78 ± 5 and 84 ± 19 \( \mu \)M, 45 ± 1 and 54 ± 5 pmol/min/mg for ND-Z and NO-Z generation, respectively. Ketoconazole (CYP3A inhibitor) inhibited ~40% of the generation of both metabolites, sulfaphenazole (CYP2C inhibitor) inhibited the formation of ND-Z, whereas \( \alpha \)-naphthoflavone (CYP1A2), quinidine (CYP2D6), and chlorzoxazone (CYP2E1) did not affect zopiclone metabolism. The generation of ND-Z and NO-Z were highly correlated to testosterone 6β-hydroxylation (CYP3A activity, \( r = 0.95 \) and 0.92, respectively; \( p = .0001 \)), and ND-Z was highly correlated to CYP2C8 activity (paclitaxel 6α-hydroxylase; \( r = 0.76, p = .004 \)). Recombinant CYP2C8 had the highest enzymatic activity toward zopiclone metabolism into both its metabolites, followed by CYP2C9 and 3A4. CYP3A4 is the major enzyme involved in zopiclone metabolism in vitro, and CYP2C8 contributes significantly to ND-Z formation.

Zopiclone is a γ-aminobutyric acid receptor agonist that is widely prescribed for its hypnotic properties (Noble et al., 1998) in insomniac patients. As benzodiazepines agents, the duration of its pharmacological effect and the occurrence of side effects, such as morning hypnotic residual effects (Allain et al., 1991), are mainly dependent on its biological half-life and clearance.

In humans, zopiclone elimination is mainly dependent on its hepatic clearance because only 5% of the drug is excreted unchanged in the urine (Noble et al., 1998). Indeed, zopiclone is extensively metabolized by the human liver into two major metabolites (Fig. 1): N-desmethyl-zopiclone (ND-Z) and N-oxide-zopiclone (NO-Z), which retain a low pharmacologic activity; and N-desmethyl-zopiclone (ND-Z), which is pharmacologically inactive (Gaillet et al., 1982, 1983; Le Liboux et al., 1987). The enzymes involved in zopiclone metabolism have not yet been identified (Noble et al., 1998), but cytochrome P-450 (CYP) isoforms may be suspected because drug interactions in humans with CYP inhibitors or inducers have been reported (Aranko et al., 1994; Jalava et al., 1996; Villikki et al., 1997). Because the pharmacological effects of this drug and its morning residual hypnotic effects may be modulated by some other drugs known to interfere with CYP activity and expression, it is important to identify the enzymes involved in zopiclone metabolism to predict and to prevent some drug interactions in humans. Therefore, the aim of the present study was to identify the human CYP isoforms involved in zopiclone metabolism in vitro.

Materials and Methods

Drugs, Chemicals, and Reagents. Zopiclone, NO-Z, and ND-Z were kindly provided by Rhône-Poulenc Rorer (Antony, France) and ketoconazole by Jansen (Beerse, Belgium). Hydroquinidine was purchased from Fluka (Buchs, Switzerland), and sulfaphenazole, quinidine, chlorzoxazone, and \( \alpha \)-naphthoflavone were obtained from Sigma Chemical Co. (St. Louis, MO). Glucose 6-phosphate, glucose 6-phosphate deshydrogenase, and NADP were purchased from Boehringer Mannheim (Meylan, France); reagents for protein assays were obtained from Pierce Chemical Co. (Beigergain, the Netherlands). All the other reagents and solvents were of the highest grade commercially available.

Human Liver Microsomes. Human liver microsomes from 12 different donors were provided by Gentest (Woburn, MA). Additional microsomes were prepared from liver samples of 10 human donors, collected, and stored as described previously (Becquemont et al., 1998).

Yeast-Expressed Recombinant Human CYP (rH-CYP) Enzymes. Human CYP 1A2, 2C8, 2C9, 2C18, 2C19, 2D6, 2E1, and 3A4 were cloned and expressed in yeast strains that overexpress endogenous NADPH-P-450 reductase, as described previously (Gautier et al., 1994; Jalava et al., 1996; Villikki et al., 1997). Because the pharmacological effects of this drug and its morning residual hypnotic effects may be modulated by some other drugs known to interfere with CYP activity and expression, it is important to identify the enzymes involved in zopiclone metabolism to predict and to prevent some drug interactions in humans. Therefore, the aim of the present study was to identify the human CYP isoforms involved in zopiclone metabolism in vitro.

Send reprint requests to: Dr. Laurent Becquemont, Faculté de Médecine, Saint Antoine Paris VI, Service de Pharmacologie, 27 Rue de Chaligny, 75012 Paris, France. E-mail: becquemo@b3e.jussieu.fr
N-desmethy-zopiclone

N-oxide-zopiclone

FIG. 1. Pathways of zopiclone metabolism in humans.

Zopiclone Metabolism. The kinetics of zopiclone oxidation and demethylation were studied in the presence of 1 mg of human liver microsomes or 100 pmol of the different rH-CYP isoforms in a final volume of 1 ml. Zopiclone was used at eight different concentrations ranging from 5 to 400 μM. Each incubation was carried out at 37°C in Tris-EDTA buffer in the presence of an NADPH-generating system consisting of 0.15 mM NADP, 2.5 mM glucose 6-phosphate, and 1.7 U/ml glucose 6-phosphate dehydrogenase. After 5-min preincubation, the reaction was started by adding the glucose 6-phosphate, and 1.7 U/ml glucose 6-phosphate dehydrogenase. After 5-min incubation was carried out at 37°C in Tris-EDTA buffer in the presence of an NADPH-generating system consisting of 0.15 mM NADP, 2.5 mM glucose 6-phosphate, and 1.7 U/ml glucose 6-phosphate dehydrogenase. After 5-min preincubation, the reaction was started by adding the glucose 6-phosphate dehydrogenase and stopped 60 min later on ice and by adding 500 μl of the HPLC mobile phase. HPLC analysis was performed on a 4.6 × 250-mm Symmetry C18 column (Waters, Milford, CT). Fluorescence detection was performed with an excitation wavelength of 300 nm and an emission wavelength of 470 nm. The isocratic mobile phase, consisting of 50 mM NaH2PO4 (pH 3.7) and acetonitrile 80:20 (v/v), was maintained at 1 ml/min during 30 min. The quantification limit of the method was 10 nM for ND-Z and 30 nM for NO-Z formation rates, respectively, from one donor to another. We observed that zopiclone was metabolized into NO-Z and ND-Z in all of the liver microsomes from the 22 different human donors. There was a 45- and 30-fold extent variability in ND-Z and NO-Z generation rate, respectively, from one donor to another. We determined the enzymatic kinetics of zopiclone metabolism in two liver samples that were chosen among the 22 liver samples for their predetermined CYP3A activity. One liver had an intermediate CYP3A activity and the other showed the lowest CYP3A activity. Enzymatic constants are presented in Table 1 and illustrated in Fig. 2. These two liver samples were found to have medium and very low turnover numbers toward the generation of both zopiclone metabolites (Fig. 3).

Intrinsic clearance (Vmax/Km) of ND-Z and NO-Z were, respectively, 10- and 3-fold lower in the liver with the lowest CYP3A activity compared with the liver with medium CYP3A activity. In both liver samples, the NO-Z and ND-Z formation rates were found to be monophasic on Eadie-Hofstee plots (Fig. 2), suggesting that a single enzyme mainly contributed to their respective generation. Furthermore, among the 22 liver samples, the generation of both
metabolites was correlated, indicating that their metabolism may be performed by the same enzyme (Fig. 3).

Screening of Zopiclone Metabolism with rH-CYP. To identify the CYP isoform(s) involved in zopiclone metabolism, we incubated zopiclone (50 μM) with a panel of rH-CYP (Fig. 4). CYP2C8 was the isoform that displayed the highest enzyme activity for the formation rates of both zopiclone metabolites (Fig. 4A). However, when we calculated from these data the expected contribution of each CYP isoform in human liver microsomes the metabolism of zopiclone (Fig. 4B), we observed that CYP3A4 was the major enzyme involved in the NO-Z formation rate, followed by CYP2C9 and CYP2C8, whereas CYP2C8 remained the major CYP isoform involved in ND-Z, followed by CYP2C9 and CYP3A4.

Determination of zopiclone enzymatic kinetic constants could be obtained from rH-CYP2C8 (Fig. 5). Apparent $K_m$ and $V_m$ reached, respectively, $71 \pm 6 \mu M$ and $2.5 \pm 0.1 \text{pmol/min/pmol CYP2C8}$ for ND-Z generation and $59 \pm 9 \mu M$ and $1.0 \pm 0.1 \text{pmol/min/pmol CYP2C8}$ for NO-Z generation. When the intrinsic clearance of ND-Z ($0.035 \text{μl/min/pmol CYP2C8}$) was extrapolated to human liver, assuming that 1 mg of human liver microsome contains an average of 10 pmol of CYP2C8, it was found to represent ~60% of the intrinsic clearance obtained in the liver sample with medium CYP3A activity (0.35 versus 0.58 μl/min/mg). NO-Z intrinsic clearance (0.017 μl/min/pmol CYP2C8) obtained from recombinant CYP2C8, when extrapolated to human liver, represented 26% of the intrinsic clearance obtained in the liver sample with medium CYP3A activity (0.17 versus 0.64 μl/min/mg). Similar determinations could not be obtained from rH-CYP3A4 because of the low turnover number of this isoform toward the generation of both zopiclone metabolites.

Effects of CYP Inhibitors on Zopiclone Metabolism in Human Liver Microsomes. To clarify the contribution of the CYP isoforms outlined previously, we incubated zopiclone with three different human liver microsomes in the presence of different prototypic CYP inhibitors (Fig. 6). ND-Z generation was mainly inhibited by ketoconazole and sulfaphenazole, whereas NO-Z generation was only significantly inhibited by ketoconazole, suggesting the involvement of CYP3A and CYP2C in ND-Z formation and CYP3A in NO-Z formation (Table 1). Surprisingly, α-naphthoflavone, a CYP3A activator, did not increase the generation of zopiclone metabolites. CYP1A, CYP2D6, and CYP2E1 inhibitors had only minor effects on the extent of zopiclone metabolism.

Correlation of Zopiclone Metabolite Generation to Different CYP Enzymatic Activities in Human Liver Microsomes. To confirm the previous results, we correlated among the different human liver samples the generation of both zopiclone metabolites to classic CYP activities. We first observed for both metabolites a unique significant correlation with testosterone 6-β-hydroxylation (CYP3A activity) when zopiclone was incubated with the 22 liver samples at a final concentration of 200 μM ($r = 0.965$ and $r = 0.859$ for ND-Z and NO-Z, respectively; $p = .0001$; data not shown). Because zopiclone concentrations in humans never reach such high levels, we performed the same experiment at a more relevant concentration (25 μM; Table 2). The generation of both metabolites was always highly correlated to CYP3A4 activity, but the ND-Z formation rate was also correlated to CYP2C8 activity.
Discussion

In the present study, we report for the first time the in vitro metabolism of zopiclone in the presence of human liver microsomes and rH-CYP. We observed that CYP3A4 was the major enzyme involved in the generation of both zopiclone metabolites and that CYP2C8 was also involved in the ND-Z formation rate.

To obtain these results, we used the three in vitro main approaches, which can be used to determine the enzymes involved in the metabolism of a drug (Rodrigues, 1994): 1) selective inhibition of the main CYP enzymatic activities in human liver microsomes with specific CYP inhibitors; 2) correlation of the generation rates of the metabolites to classic predetermined CYP activities in different human liver samples; and 3) screening the metabolism of the drug with a panel of different heterologously expressed human CYP.

Zopiclone Metabolism in Human Liver Microsomes. We observed that zopiclone was metabolized into two major metabolites, ND-Z and NO-Z; this is consistent with the observation in humans (Goa and Heel, 1986). There was a very large interindividual variability in zopiclone metabolism from one liver sample to another, a characteristic that is in agreement with most of the drugs metabolized by CYP in humans and with the large variability of expression of most of the different human liver CYPs (Guengerich and Turvy, 1991; Shimada et al., 1994).

The metabolism of zopiclone into its two metabolites was found to be monophasic on Eadie-Hofstee plots, suggesting that its metabolism was probably mainly dependent on a single enzyme. CYP3A seemed to influence the generation of both zopiclone metabolites because the liver sample with the lowest CYP3A activity had a 10- and 3-fold

![Graph A](rH-CYP)

![Graph B](rH-CYP)

![Graph C](Materials and Methods)

![Graph D](Materials and Methods)

![Graph E](Materials and Methods)

![Graph F](Materials and Methods)
lower intrinsic clearance of ND-Z and NO-Z, respectively, compared with another liver sample with a medium CYP3A activity.

CYP inhibitors indicated that CYP3A was responsible for at least 40% of the metabolism of zopiclone into its two metabolites and that the CYP2C subfamily accounted for about 40% of ND-Z formation. Correlation studies confirmed the major involvement of CYP3A4 in both zopiclone metabolites generation and the significant contribution of CYP2C8 in ND-Z formation.

**Zopiclone Metabolism in the Presence of rH-CYP.** Screening of human liver microsomes with a panel of different heterologously expressed human CYPs indicated that CYP2C8 had the highest enzymatic activity for the generation of both zopiclone metabolites. These results were adjusted to the relative content of the different CYP isoforms in human liver microsomes and indicated that: 1) CYP3A4 could be identified as the major isoform involved in NO-Z formation but not in ND-Z formation; 2) CYP2C8 was found to be the major CYP in ND-Z formation and had a lower contribution to NO-Z generation; and 3) CYP2C9 contributed significantly to the formation of both metabolites. However, our results cannot exclude the concomitant participation of other CYP isoforms, such as CYP2A6 and CYP2B6 or flavin monoxygenases, in zopiclone metabolism.

**References**


