EFFECT OF MULTIPLE DOSING OF KETOCONAZOLE ON PHARMACOKINETICS OF MIDAZOLAM, A CYTOCHROME P-450 3A SUBSTRATE IN BEAGLE DOGS

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ABSTRACT:
To evaluate effects of multiple dosing of ketoconazole (KTZ) on hepatic CYP3A, the pharmacokinetics of intravenous midazolam (MDZ, 0.5 mg/kg) before and during multiple dosing of KTZ were investigated in beagle dogs. KTZ tablets were given orally to dogs (n = 4) for 30 days (200 mg b.i.d.). With coadministration of KTZ, t1/2 of MDZ were significantly increased both on day 1 (2-fold) and on day 30 (3-fold). Total body clearance (CLtot) of MDZ declined gradually during the first 5 days after the start of KTZ treatment, and thereafter CLtot appeared to reach a plateau phase (one-fourth), depending on plasma KTZ concentrations. The effects of KTZ on the biotransformation of MDZ were also investigated using dog liver microsomes (n = 5). The Ki values of KTZ for MDZ 1'-hydroxylation and 4-hydroxylation were 0.0237 and 0.111 μM, respectively, indicating that KTZ extensively inhibits hepatic CYP3A activity in dogs. CLtot values estimated from in vitro Ki values corrected by unbound fraction of KTZ and unbound concentrations of the drug in plasma were consistent with in vivo CLtot of MDZ. The results in this study suggest that KTZ treatment is necessary until plasma concentrations of the drug reach a steady state to evaluate the effect of multiple dosing of the drug on hepatic CYP3A in vivo. In addition, it is suggested that Ki values corrected by unbound fraction of KTZ and unbound concentrations of the drug in plasma enable precise in vitro-in vivo scaling.

In human liver and small intestine, cytochrome P-450 3A (CYP3A) is the most important subfamily among the cytochrome P-450 superfamily. CYP3A catalyzes the biotransformation of a wide variety of exogenous and endogenous substances (Guengerich, 1999) and plays a significant role in the metabolism of about half of the available drugs (Guengerich, 1995). Because of the large number of drugs metabolized by CYP3A, the potential for drug-drug interactions to occur is substantial (Dresser et al., 2000). Drug-drug interactions may cause serious adverse effects in clinical practices. For example, case reports of drug-drug interactions resulting in adverse effects have been published for felodipine and erythromycin (Bailey et al., 1996), lovastatin and itraconazole (Lees and Lees, 1995), and cisapride and diltiazem (Thomas et al., 1998).

The antymiotic agent ketoconazole (KTZ1) is one of the potent CYP3A inhibitors (Albengres et al., 1998; Lomaestro and Piatek, 1998). Its inhibitory effects in vitro on metabolic activity of CYP3A using liver microsomes have been well studied by many investigators. They have demonstrated that KTZ is the most potent CYP3A inhibitor among all the CYP3A inhibitors tested (Newton et al., 1995; von Molkke et al., 1996; Wang et al., 1999). It has also been reported that KTZ causes clinically relevant interactions with different CYP3A substrates, including cyclosporine (Gomez et al., 1995), tacrolimus (Floren et al., 1997), and terfenadine (Honig et al., 1993). However, only few studies have been performed on the change of the decrease in CYP3A metabolic activity when KTZ is given by multiple dosing over a long term (Venkatakrishnan et al., 2000). KTZ has to be given chronically in clinical cases.

In the present study, we examined in dogs the effects of multiple oral dosing of KTZ on in vivo hepatic CYP3A activity by determining the intravenous pharmacokinetics of midazolam (MDZ), a classical probe for CYP3A activity (Thummel et al., 1994a,b). Moreover, we investigated the effects of KTZ on the biotransformation of MDZ using dog liver microsomes to quantify the inhibitory effects of KTZ on CYP3A metabolic activity and to examine whether the in vivo drug-drug interaction was quantitatively predictable even in the situation that the interacting drug was administered over a long term. We selected beagle dogs as an animal model because the animals have been extensively used to investigate metabolism of xenobiotics both in vivo and in vitro and the plasma concentration-time profile of KTZ after oral administration in dogs is similar to that in humans, although body weight-standardized dose is different between the species (Baxter et al., 1986).

Experimental Procedures

Materials. KTZ was purchased as a tablet (Nizoral) from Janssen Pharcmeutica (Titusville, NJ) and as a reagent from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). MDZ and the metabolites, 1'-OH MDZ and 4-OH MDZ, were obtained from Daiichi Pure Chemicals Co., Ltd. (Tokyo, Japan) and a MDZ injectable solution (Dormicam) was from Yamanouchi Pharmaceutical Co. (Tokyo, Japan). Diazepam was obtained as a reagent from Sigma (St. Louis, MO). All other chemicals used as reagents were of analytical and HPLC grade.

Animals. Nine beagle dogs (male, 1-year-old) weighing 10 to 15 kg were
obtained from CSK Research Park Co. Ltd. (Nagano, Japan). Four of nine dogs were used for in vivo studies; five were used for in vitro studies. The dogs were allowed access to water ad libitum and were given food twice a day (8 AM and 8 PM).

**In Vivo Studies. Study design.** Four beagle dogs were used to investigate the effects of multiple dosing of KTZ on hepatic CYP3A activity. KTZ was given orally to the dogs 1 h after feeding for 30 days (200 mg/body b.i.d.; 9 AM and 9 PM). MDZ (0.5 mg/kg) was intravenously administered 7 days before the beginning of multiple dosing of KTZ (day 0 as control) and 1 h after KTZ administration on the morning of days 1, 2, 3, 5, 8, 12, 19, and 30 after the beginning of the KTZ treatment to evaluate hepatic CYP3A activity in vivo by means of total body clearance (CL_{T,B}) of MDZ (Thummel et al., 1994a,b).

**Blood sampling.** Blood samples (2.5 ml) were collected at 0.25, 0.5, 0.75, 1, 1.25, 1.5, 1.75, and 2 h (on days 1, 2, 3, and 2 or at 0.25, 0.5, 0.75, 1, 1.5, 2, 3, and 4 h (on days 8, 12, 19, and 30) after MDZ injection for measurement of plasma MDZ concentrations. According to the previous study (Baxter et al., 1986) and our preliminary study on pharmacokinetics of KTZ in beagle dogs after single oral administration of KTZ (data not shown), blood samples (1 ml) were obtained at 3 and 12 h after KTZ administration for the determination of peak and trough concentrations of KTZ in plasma. Plasma was separated from whole blood by centrifugation and stored at −80°C before HPLC analysis.

**In Vitro Studies. Preparation of dog liver microsomes.** Five beagle dogs were euthanized by intravenous pentobarbital injection (25 mg/kg) to obtain liver samples. The microsomal fractions were prepared as described by van der Hoeven and Coon (1974). The obtained samples were frozen at −80°C until use. The protein levels and cytochrome P-450 contents were determined as described by Lowry et al. (1951) and Omura and Sato (1964), respectively.

**Enzyme kinetic analysis.** The kinetic and inhibition studies for MDZ in dog liver microsomes were performed on the incubation condition described as follows: 0.23 ml of incubation mixture containing dog liver microsomes (approximately 0.5 mg/ml) and an NADPH generating system [50 mM phosphate buffer (pH 7.4), 0.5 mM NADP, 5 mM glucose 6-phosphate, 0.4 U glucose-6-phosphate dehydrogenase, and 5 mM MgCl₂] were preincubated at 37°C for 5 min. Varying quantities of MDZ in 1% methanol solution, to yield final incubate concentrations ranging from 6.14 to 368 μM, were added to a series of incubation tubes. For inhibition studies, incubations were also performed with coaddition of KTZ in ethanol solution (final concentration 4.7, 9.4, and 18.8 μM) because preliminary studies showed that the KTZ concentrations were more suitable than the others for estimating inhibition constant (Kᵢ). Formation of metabolites was linear with respect to incubation time (0–10 min) and microsomal protein concentration (0–0.5 mg/ml). The reactions were initiated by adding 10 μl of MDZ solution and 10 μl of ethanol (without KTZ) or KTZ solution. The final reaction volume was 250 μl. After incubation at 37°C for 10 min, the enzyme reactions were terminated with 100 μl of acetonitrile and placed on ice. After centrifugation at 3000g for 5 min, the resulting supernatant was immediately applied to HPLC system.

**Microsomal protein binding of MDZ and KTZ.** To obtain unbound concentrations of MDZ and KTZ in the assay system, the binding of the drugs to microsomal protein was determined. One milliliter of the mixture as the same in enzyme kinetic analysis except NADP was incubated at 37°C for 30 min and transferred to a device of ultrafiltration kit (Centrifree; Amicon, Beverly, MA), followed by centrifugation at 2000g for 5 min. The resulting ultrafiltrate was immediately analyzed to obtain unbound concentrations of MDZ and KTZ.

**Data Analysis. Pharmacokinetic parameters after intravenous MDZ administrations.** Plasma concentration-time curves after MDZ injection were fitted to the following biexponential equation using the nonlinear least-squares regression (Yamaoka et al., 1981).

\[ C_p = A \times e^{-\frac{t}{\tau_{1/2}}} + B \times e^{-\frac{t}{\tau_{1}}} \]  

(1)

Also, pharmacokinetic parameters including half-life (τ₁/₂), C₀, and volume of distribution at steady state (Vd₀) were calculated using conventional methods.

The kinetic parameters for metabolism of MDZ and inhibition by KTZ in dog liver microsomes. As previously reported in humans and mouse (von Moltke et al., 1996; Warrington et al., 2000), formation of 4-OH MDZ from MDZ without KTZ was consistent with single-enzyme Michaelis-Menten kinetics, and the formation with KTZ was consistent with single-enzyme Michaelis-Menten kinetics and competitive inhibition (Fig. 3A). Accordingly, the following equations were fitted to the observed data using the nonlinear least-squares regression (Yamaoka et al., 1981) to estimate kinetic parameters for hydroxylation of MDZ and inhibition by KTZ in dog liver microsomes:
where \( v \) is the velocity of 4-OH MDZ formation, and \( S \) and \( I \) are the unbound concentration of substrate, MDZ, and inhibitor, KTZ, respectively. \( V_{max} \), \( K_m \), and \( K_i \) represent maximal metabolic reaction velocity, Michaelis-Menten constant, and inhibition constant, respectively. \( K_m \) and \( K_i \) were calculated from unbound concentrations of MDZ and KTZ, respectively.

The pattern of 1'-OH MDZ formation from MDZ was consistent with Michaelis-Menten kinetics with competitive substrate inhibition, and the inhibition by KTZ showed competitive inhibition (von Moltke et al., 1996; Warrington et al., 2000) (Fig. 3B). The following equations were fitted to 1'-OH MDZ data points:

\[
\frac{v}{K_m + S} + \frac{V_{max} \times S}{K_m \times (1 + I/K_i)} + \frac{S}{1 + I/K_i}
\]

where \( v, S, I, V_{max}, K_m, \) and \( K_i \) are as described above, and \( K_s \) is the substrate inhibition constant.

The prediction of inhibitory effect of KTZ on in vivo clearance of MDZ. The decreasing ratio of intrinsic clearance both for 1'-hydroxylation and for 4-hydroxylation were estimated from in vitro data using the following equation:

\[
R = \frac{CL_{int1}}{CL_{int2}} = \frac{1}{1 + I/K_i}
\]

where \( CL_{int1} \) and \( CL_{int2} \) are intrinsic clearance without and with KTZ, respectively. The inhibitor concentrations (\( I \)) were calculated by multiplying average concentrations of KTZ on each experimental day.

Each point represents the mean ± S.D. (\( n = 4 \)). Lines were calculated by the nonlinear least-squares regression analysis. * indicates significant differences between the parameters on day 0 and the others by the Student’s t-test \( (p < 0.05) \), ‡ indicates significant differences between the parameters on day 1 and 30 by the Student’s t-test \( (p < 0.05) \).

**Results**

Effects of Multiple Oral Dosing of KTZ on the Pharmacokinetics of i.v. MDZ in Dogs. Figure 1 shows plasma concentration-time profiles of MDZ after intravenous administrations on day 0 (without KTZ), 1 (corresponding to one single dosing of KTZ), and 30 (after the last dose of KTZ). Plasma MDZ concentrations increased by oral KTZ administration. The extent on day 30 was much larger than that on day 1. The pharmacokinetic parameters for MDZ on day 0, 1, and 30 are represented in Table 1. With coadministration of KTZ, the \( t_{1/2B} \) for MDZ significantly increased both on day 1 (2-fold) and on day 30 (3-fold). Statistically significant decreases of \( CL_{int} \) were observed both on day 1 (one-half) and on day 30 (one-fourth). Compared with the parameters on day 1, \( t_{1/2B} \) on day 30 was 2-fold larger whereas \( CL_{int} \) was halved. \( V_{max} \) for MDZ did not change. Figure 2 shows the changes in \( CL_{int} \) for MDZ after the start of multiple oral dosing of KTZ. \( CL_{int} \) for MDZ declined gradually during the first 5 days, and thereafter it appeared to reach a plateau phase at about 10 ml/min/kg. During the experiment, significant decreases in \( CL_{int} \) were observed.

The Profiles and Kinetic Parameters for the Biotransformation of MDZ and the Inhibition by KTZ in Dog Liver Microsomes. Figure 3 shows the profiles for 1'-hydroxylation and 4-hydroxylation of MDZ and the inhibition by KTZ in microsomes prepared from a representative dog liver. KTZ competitively inhibited both pathways of MDZ metabolism. Table 2 shows the kinetic parameters for biotransformation of MDZ and the inhibition by KTZ in dog liver microsomes. The mean value of \( K_m \) for formation of 1'-OH MDZ was 4.84 μM, with a high affinity. The \( K_s \) (substrate inhibition constant) values averaged 443 μM, and ranged from 275 to 704 μM. The mean value of \( K_m \) for formation of 4-OH MDZ was approximately 5-fold lower.
Effects of multiple oral dosing of KTZ (200mg b.i.d.) on pharmacokinetic parameters of MDZ (0.5mg/kg) after intravenous administrations to beagle dogs.

<table>
<thead>
<tr>
<th>Day</th>
<th>0</th>
<th>1</th>
<th>30</th>
</tr>
</thead>
<tbody>
<tr>
<td>$t_{1/2p}$ (h)</td>
<td>0.558 ± 0.118</td>
<td>1.20 ± 0.56*</td>
<td>2.10 ± 0.43*</td>
</tr>
<tr>
<td>$CL_{int}$ (ml/min/kg)</td>
<td>35.2 ± 2.8</td>
<td>20.9 ± 9.4*</td>
<td>10.2 ± 4.5*†</td>
</tr>
<tr>
<td>$Vd_{ss}$ (l/kg)</td>
<td>1.07 ± 0.17</td>
<td>1.25 ± 0.57</td>
<td>1.27 ± 0.34</td>
</tr>
</tbody>
</table>

* Significant differences between the parameters on day 0 and the others by the paired Student’s t test (p < 0.05).
† Significant differences between the parameters on day 1 and 30 by the paired Student’s t test (p < 0.05).

TABLE 2

Kinetic parameters for the biotransformation of MDZ and inhibition by KTZ in dog microsomes

<table>
<thead>
<tr>
<th>$V_{max}$ (nmol/min/mg protein)</th>
<th>$K_m$</th>
<th>$K_i$</th>
<th>$K_s$</th>
</tr>
</thead>
<tbody>
<tr>
<td>1'-OH</td>
<td>1.21 ± 0.45</td>
<td>4.84 ± 2.86</td>
<td>443 ± 177</td>
</tr>
<tr>
<td>4-OH</td>
<td>0.587 ± 0.305</td>
<td>26.7 ± 18.3</td>
<td>0.111 ± 0.046</td>
</tr>
</tbody>
</table>

The purpose of this study was to investigate the effects of multiple oral dosing of KTZ on hepatic CYP3A activity because KTZ is chronically administered to patients. MDZ was intravenously administered to assess hepatic CYP3A activity (Thummel et al., 1994a,b). Moreover, it was tested by inhibition study using liver microsomes to determine whether the drug-drug interaction was quantitatively predictable even in the situation that the interacting drug was administered over a long term.

Plasma concentration-time profiles of i.v. MDZ in dogs were marked larger than that of 1’-OH. These results show that $CL_{int}$ for 1’-hydroxylation accounted for more than 90% of the sum of both pathways. The $K_i$ values for both pathways were approximately 100-fold lower than those of $K_m$, indicating that KTZ possesses potent inhibitory effects on both pathways of MDZ metabolism in dogs.

The Prediction of Inhibitory Effect of KTZ on in Vivo Clearance of MDZ. Average concentrations of KTZ in plasma on day 30 ranged from 3.64 to 19.1 μg/ml (Fig. 4). Using these values and unbound fractions of KTZ ($fu = 0.032$), plasma unbound concentrations of KTZ were obtained (0.219–1.15 μM). The ratio of intrinsic clearance on day 30 after the beginning of multiple dosing of KTZ ($CL_{int}^{}\star$) to that without KTZ ($CL_{int}$) calculated from eq. 6 are presented in Table 3. $CL_{int}$ for formation from MDZ to 1’-OH MDZ decreased to about 10- to 50-fold by KTZ, whereas that for 4-OH MDZ decreased to about 3- to 10-fold. Therefore, the total intrinsic clearance of MDZ metabolism decreased to about 9- to 40-fold by KTZ. The profiles of $CL_{int}$ of MDZ predicted from in vitro data ($CL_{tot}$ in vitro) and the observed $CL_{tot}$ in vivo ($CL_{tot}$ in vivo) are shown in Fig. 5. The plot of the $CL_{tot}$ in vitro values against $CL_{tot}$ in vivo values is also represented in Fig. 6. Both Figs. 5 and 6 shows that the $CL_{tot}$ in vivo and $CL_{tot}$ in vitro were almost comparable.

Discussion

The change in $CL_{tot}$ of MDZ after the start of multiple oral dosing...
of KTZ was investigated in this study. $\text{CL}_{\text{tot}}$ of MDZ decreased to 30% during the first 5 days, and thereafter reached a plateau phase (Fig. 2). Peak concentrations of KTZ in plasma also reached a steady state at about 5 days after the start of KTZ administrations (Fig. 4). These results show that the extent of inhibition of in vivo CYP3A activity depends on plasma KTZ concentration, suggesting that its inhibition by oral KTZ administration may be accounted for only by competitive inhibition on hepatic CYP3A. Therefore, KTZ treatment may be necessary until plasma concentration of the drug reaches a steady state to evaluate the effect of multiple dosing of the drug on hepatic CYP3A in vivo in humans and in dogs.

Because data of the effect of multiple oral dosing of KTZ on pharmacokinetics of i.v. MDZ in humans are lacking, the result on day 1 in this study was compared with that in humans described by Tsunoda et al. (1999). Plasma KTZ concentrations on day 1 in this study were close to those in the human study. The single dose of KTZ decreased $\text{CL}_{\text{tot}}$ of MDZ to 20% in humans and to 50% in dogs (Fig. 2). However, this comparison is incorrect because $\text{CL}_{\text{tot}}$ does not reflect directly hepatic metabolic capacity, especially in the case of drugs that represent a high extraction ratio (Ito et al., 1998), and because $\text{CL}_{\text{tot}}$ of MDZ represents a high extraction in dogs and a low extraction in humans (Tsunoda et al., 1999).

To compare the effects of KTZ on the metabolic capacity for MDZ or hepatic CYP3A activities between humans and dogs $\text{CL}_{\text{int}}$ that directly represents metabolic capacity was calculated by substituting $\text{CL}_{\text{tot}}$ to eq. 7. In this calculation, 25.4 and 42.3 ml/min/kg were used as the values of $Q$ for humans and dogs, respectively (Boxenbaum, 1980). The calculation shows that KTZ decreases $\text{CL}_{\text{tot}}$ for MDZ to 16% for humans and to 26% for dogs. For estimating $K_i$ values from eq. 6, inhibitor concentrations ($I$) were calculated by multiplying plasma KTZ concentrations (about 3 $\mu$g/ml for humans; approximately 4.4 $\mu$g/ml for dogs) by plasma unbound fraction. Substituting the calculated $R$ values and $I$ to eq. 6, it was estimated that $K_i$ values of KTZ for MDZ metabolism in humans is about 10-fold smaller than in dogs. Therefore, it is suggested that KTZ inhibits hepatic CYP3A activity more extensively in humans than in dogs.

Moreover, to quantify the inhibitory effect of KTZ on hepatic CYP3A in dogs, in vitro studies using prepared dog liver microsomes were conducted in this study. The $K_i$ values of KTZ were markedly small, indicating that KTZ strongly inhibits MDZ metabolism. Comparison of $K_i$ values of KTZ in dogs with those in humans shows that KTZ inhibits hepatic CYP3A stronger in humans than in dogs (von Mollke et al., 1996), supporting the possibility that KTZ inhibits in vivo hepatic CYP3A activity in humans more extensively than in dogs.

As shown in Fig. 5, $\text{CL}_{\text{tot}}$ values estimated from in vitro data were consistent with in vivo $\text{CL}_{\text{tot}}$ of MDZ. This success may be attributed to the following: 1) we used unbound $K_i$ values based on Michaelis-Menten theory but not $K_i$ values calculated from total concentration of KTZ, and 2) we also used unbound concentrations of KTZ in plasma as $I$ on the assumption that the drug entered hepatocytes only by passive diffusion because the drug has not been reported to be actively transported from plasma into hepatocytes. von Mollke et al. (1998) successfully predicted in vivo inhibition of MDZ clearance by KTZ using total plasma concentration and $K_i$ values calculated from total concentration of KTZ. This success may be due to the similarity of unbound fraction of KTZ in an assay system containing microsomal proteins to that in plasma. In many cases, however, the similarity seems to be rare. Therefore, plasma unbound concentration of inhibitor and $K_i$ values corrected by unbound fraction of inhibitor may result in precise in vitro-in vivo scaling in case of inhibitor that may be not actively transported into the liver.

In summary, multiple oral dosing of KTZ to dogs gradually decreased the $\text{CL}_{\text{tot}}$ of MDZ during the first 5 days, and thereafter $\text{CL}_{\text{tot}}$ almost stabilized during experimental period. These changes depended on plasma KTZ concentration, suggesting that its inhibition by oral KTZ administration may be accounted for only by competitive inhibition on hepatic CYP3A. Therefore, KTZ treatment may be necessary until plasma concentration of the drug reaches a steady state to evaluate the effect of multiple dosing of the drug on hepatic

### Table 3

<table>
<thead>
<tr>
<th>Metabolic pathway</th>
<th>$R$</th>
</tr>
</thead>
<tbody>
<tr>
<td>1′-OH</td>
<td>0.020–0.091</td>
</tr>
<tr>
<td>4-OH</td>
<td>0.085–0.32</td>
</tr>
<tr>
<td>Total*</td>
<td>0.025–0.11</td>
</tr>
</tbody>
</table>

* Sum of intrinsic clearance for both pathways.
CYP3A in vivo in humans and dogs. In vitro-in vivo scaling of KTZ inhibition was examined using Ki values corrected by unbound fraction of KTZ and unbound concentrations of the drug in plasma. CL\text{out} of MDZ estimated from in vitro data was consistent with in vivo CL\text{out}. For precise in vitro-in vivo scaling also in humans, we recommend use of Ki values corrected by unbound fraction of KTZ and unbound concentrations of the drug in plasma but not total concentration.

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References


