Alprazolam as an \textit{in vivo} Probe for Studying Induction of CYP3A in Cynomolgus Monkeys

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Running title page

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ABBREVIATIONS: CYP, cytochrome P450; APZ, alprazolam; MDZ, midazolam; KTZ, ketoconazole; RIF, rifampicin; DDI, drug-drug interaction; AUC, area under the plasma concentration-time curve; Cmax, maximum plasma concentration; Tmax, time to reach Cmax.
Abstract

Induction of the cytochrome P450 (CYP) enzyme is a major concern in the drug discovery processes. In order to predict the clinical significance of enzyme induction, it is helpful to investigate pharmacokinetic alterations of a co-administered drug in a suitable animal model. In this study, we focus on the induction of CYP3A, which is involved in the metabolism of approximately 50% of marketed drugs and is inducible in both the liver and intestine. As a marker substrate for CYP3A activity, alprazolam (APZ) was selected and characterized using recombinant CYP3A enzymes expressed in *Escherichia coli*. Both human CYP3A4 and its cynomolgus P450 ortholog predominantly catalyzed APZ 4-hydroxylation with sigmoidal kinetics. When administered intravenously and orally to cynomolgus monkeys, APZ had a moderate clearance; its first-pass extraction ratio after oral dosing was estimated to be 0.09 in the liver and 0.45 in the intestine. Pretreatment with multiple doses of rifampicin (20 mg/kg p.o. for 5 days), a known CYP3A inducer, significantly decreased plasma concentrations of APZ after intravenous and oral administrations (0.5 mg/kg), and first-pass extraction ratios were increased to 0.39 in the liver and 0.63 in the intestine. The results were comparable to those obtained in clinical DDI reports related to CYP3A induction, although the rate of recovery of CYP3A activity seemed to be slower compared with those estimated in clinical studies. In conclusion, pharmacokinetic studies using APZ as a probe in monkeys may provide useful information regarding the prediction of clinical DDIs due to CYP3A induction.
Introduction

The CYP3A subfamily, the most abundant P450 enzymes in the human liver and small intestine, is involved in the biotransformation of nearly 50% drugs that are involved in clinical use and is thus, the target of many drug-drug interactions (DDIs). *In vitro* studies with human tissue preparations have been used as screening approaches for assessing the potential of clinical DDIs in the drug discovery process. Once a moderate or weak DDI is identified, its clinical significance needs to be evaluated *in vivo* later in the development process. One useful approach is a carefully designed pharmacokinetic DDI study in experimental animals.

Monkeys are widely used in preclinical drug safety evaluation and biotransformation studies. Several studies have demonstrated that monkeys are particularly useful in interspecies scaling for predicting human pharmacokinetics (Ward and Smith, 2004; Ward et al., 2005; Sakuda et al., 2006; Ogasawara et al., 2007). Recent studies have identified a number of P450 cDNAs from cynomolgus monkeys, including those of CYP3A8 and CYP3A5, which show high sequence identities (94–95%) with the orthologous human CYP3A4 and CYP3A5, respectively (Uno et al., 2007, 2010; Iwasaki and Uno, 2009). Furthermore, when co-administered with typical CYP3A inhibitors, monkeys administered an oral dose of CYP3A substrates, such as midazolam (MDZ) and simvastatin, showed markedly higher plasma concentrations compared with those who were administered a dose of substrate alone (Kanazu et al., 2004; Ogasawara et al., 2007, 2009a, 2009b). These findings suggest that monkeys can be used to investigate the underlying mechanism of and to predict the likelihood of clinical DDIs when CYP3A inhibition is involved.
The objective of the present study is to investigate DDI related to CYP3A induction using cynomolgus monkeys as a preclinical animal model. Recently, a pharmacokinetic study in rhesus monkeys revealed that hepatic availability of MDZ was markedly reduced by pretreatment with rifampicin (RIF), a known CYP3A inducer (Prueksaritanont et al., 2006). However, the study failed to detect any increase in total clearance of MDZ following intravenous administration, because its hepatic clearance was close to hepatic blood flow rate in monkeys. Monkeys also show poor oral bioavailability for other CYP3A substrates such as verapamil (~1%), nifedipine (~1%), methotrexate (8%), and simvastatin (0.8%), which are therefore not well suited for in vivo enzyme induction study (Ogasawara et al., 2009a; Takahashi et al., 2009). In addition, CYP3A is known to be induced in the small intestine as well as in the liver, and clinical studies have shown that repeated oral administration of representative CYP3A inducers, such as RIF and St. John’s wort, resulted in the up-regulation of CYP3A expression in the small intestine (Glaeser et al., 2005; Tannergren et al., 2004) and increased intestinal first-pass metabolism of verapamil, another CYP3A substrate (Fromm et al., 1996). In this study, we used alprazolam (APZ), a known CYP3A probe in humans as well (Schmider et al., 1999), as a marker substrate for pharmacokinetic studies in monkeys. This is because APZ was metabolized primarily by CYP3A in monkeys as shown in the present study, and more importantly, because it was expected to be a moderate-clearance drug that may allow the detection of P450 induction in the intestine and liver. In addition, we investigated enzyme kinetics for the formation of 1′-hydroxy APZ and 4-hydroxy APZ using recombinant monkey and human CYP3A isoforms, expressed in Escherichia coli (E. coli), and compared the results. As recommended by the P450 Nomenclature
Committee (http://drnelson.utmem.edu/cytochromeP450), in this article, we designate cynomolgus CYP3A8 and rhesus CYP3A64, both orthologous to human CYP3A4, as CYP3A4.
Methods

Chemicals and Biologicals. APZ was purchased from Wako Pure Chemical Industries (Osaka, Japan) for the pharmacokinetic and the in vitro metabolic studies and from Sigma-Aldrich (St. Louis, MO) for the in vitro study of enzyme induction. MDZ and 1'-hydroxyalprazolam-d5 were purchased from Wako Pure Chemical Industries. 4-Hydroxyalprazolam (4-OH APZ) and 1'-hydroxyalprazolam (1'-OH APZ) were purchased from BIOMOL International (Exeter, UK). 1'-Hydroxymidazolam (1'-OH MDZ), 4-hydroxymidazolam (4-OH MDZ), and APZ-d5 were purchased from Sigma-Aldrich. RIF was purchased from Wako Pure Chemical Industries for the in vivo study and from Sigma-Aldrich for the in vitro study. Polyethylene glycol 300 was purchased from Wako Pure Chemical Industries. Pooled liver and small intestinal microsomes from humans and monkeys were supplied by Xenotech (Lenexa, KS). Recombinant P450 enzymes, co-expressed in *E. coli* membranes with human NADPH-P450 reductase, were prepared as described previously (Yamazaki et al., 2002; Uno et al., 2007). Cryopreserved hepatocytes of 2 cynomolgus monkeys were obtained from Biopredic International (Rennes, France). All other reagents and solvents were of an analytical grade and were commercially available.

Animals. Male cynomolgus monkeys, 4-6 kg, were supplied by Yulin Hongfeng Experimental Animal’s Domesticating and Breeding Center (Guangxi, China) and Guandong Zhaoqing Laboratory Animals Research Center Xiangang (Guangdong, China). Animals were housed in a temperature- and humidity-controlled room with a 12-h light/dark cycle. Animals were fed a standard animal diet (Teklad Global Certified 25% Protein Primate Diet, Harlan Sprague-Dawley, Indianapolis, IN); food was provided
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*ad libitum* except during the overnight periods before dosing. Whenever overnight fasting was employed, food was provided after the 6-h blood sample was obtained. All procedures for the animal experiments were approved by the Animal Ethics Committee of Mitsubishi Tanabe Pharma Corporation.

**In Vitro Studies Using Microsomes.** All incubations were performed in duplicate. Measurement of the formation rates of 1′-OH APZ, 4-OH APZ, 1′-OH MDZ, and 4-OH MDZ was conducted in incubation mixtures containing microsomes (0.05–0.2 mg protein/mL), an NADPH-regenerating system (1.3 mM NADP⁺, 3.3 mM glucose 6-phosphate, 3.3 mM MgCl₂, and 0.4 unit/mL glucose-6-phosphate dehydrogenase), and APZ or MDZ (10 μM) in 0.10 M potassium phosphate buffer (pH 7.4). APZ or MDZ was initially dissolved in methanol, and the final concentration of methanol in the incubation mixture was 1% (v/v). After preincubation for 15 min at 37°C, each reaction was initiated by adding the NADPH-regenerating system. After incubation at 37°C for 2–20 min, the reaction was stopped by adding 4 volumes of ice-cold acetonitrile/methanol (1:1, v/v) containing an internal standard, which was MDZ and APZ in studies on APZ and MDZ, respectively. After centrifugation, supernatants were analyzed by liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS). The incubation conditions were within the linear range for protein concentration and incubation time.

The effect of a CYP3A-selective inhibitor was investigated on APZ metabolism using ketoconazole (KTZ). KTZ was dissolved in methanol, and liver or intestine microsomes (0.25 mg protein/mL) were incubated with APZ (100 μM) in the presence of KTZ at concentrations of 0.005, 0.01, 0.05, 0.1, 0.5, 1, and 5 μM. The final concentration of the organic solvent in the incubation mixture was 1% (v/v).
**In Vitro Studies Using Recombinant P450 Isoforms.** All incubations were performed in duplicate. Measurement of the formation rates of 1'-OH APZ and 4-OH APZ was conducted in incubation mixtures containing recombinant P450, an NADPH-regenerating system (1.3 mM NADP⁺, 3.3 mM glucose 6-phosphate, 3.3 mM MgCl₂, and 0.4 unit/mL glucose-6-phosphate dehydrogenase), and APZ (2.5–1000 μM) in 0.10 M potassium phosphate buffer (pH 7.4). Incubation time (5–20 min) and P450 concentrations (10–30 pmol/mL) were within the linear range. APZ was initially dissolved in methanol, and the final concentration of methanol in the incubation media was 1% (v/v). After preincubation for 15 min at 37°C, each reaction was initiated by the addition of the NADPH-regenerating system. Reactions were terminated by the addition of ice-cold methanol/acetonitrile (1:1, v/v) containing MDZ as the internal standard. After centrifugation, supernatants were analyzed by LC-MS/MS.

**In Vitro Induction Study.** Cryopreserved hepatocytes of 2 cynomolgus monkeys were suspended in a thawing medium and plated onto collagen-coated 24-well plates (3 × 10⁵ cells/well). After the cells were maintained at 37°C for 24 h, the medium was replaced with Matrigel-containing Williams E medium. After a 48-h acclimation, hepatocytes were treated in duplicate at 37°C for 72 h with a vehicle (0.1% DMSO) or RIF (0.08–10 μM). Over the treatment period, dosing media were replaced every 24 h, and at the end of the treatment period, the medium was aspirated from wells and replaced with Williams E medium containing APZ for measuring CYP3A activity, as described below.

To measure CYP3A activity, 4-hydroxy APZ was determined after incubation of 30 μM APZ with hepatocytes in Williams E medium at 37°C, 95% humidity, and 5% CO₂ for 60 min. The medium was
collected and diluted 10-fold with Williams E. medium. Each aliquot was added to 2 volumes of methanol/acetonitrile (1:1, v/v) containing verapamil (0.2 μM) as an internal standard, and analyzed by LC-MS/MS.

**In Vivo Studies.** Three monkeys were used for 2 study periods. For first and second periods, we examined pharmacokinetics of APZ given as oral and intravenous doses, respectively. Each period was started by a single dose of APZ without RIF, and followed by multiple dosing of RIF with a subsequent recovery phase. The second period was started 2 weeks after the end of the first period. The dosing solution of APZ was prepared in saline containing 9% (w/v) polyethylene glycol 300, and administered to overnight fasted animals at 0.5 mg/kg orally by gavage or intravenously by a bolus injection into the femoral vein. RIF was dissolved in 0.1 M HCl and diluted 10-fold with saline to obtain a dosing solution at a concentration of 4 mg/mL, which was administered orally by gavage. At the start of each study period, the vehicle solution was administered before APZ dosing. After 1 week, animals received RIF (20 mg/kg, p.o.) once daily for 5 days. The last dose of RIF was given immediately before APZ dosing. APZ was also administered 1 day, 1 week, 4 weeks, and 11 weeks after the last dose of RIF. Whenever APZ was administered, blood samples were collected from the femoral vein at pre-dose and at 15 and 30 min intervals as well as at 1, 2, 4, 6, 8, and 24 h after APZ dosing. Blood was also collected 5 min after intravenous dosing of APZ. After centrifugation of the blood, plasma was mixed with an equal volume of ascorbic acid (200 μg/mL).

**Analytical Procedures.** *Determination of 4-OH APZ concentrations in an in vitro study of CYP3A*
induction. The concentrations of 4-OH APZ in samples of in vitro induction studies were measured using a 2795 high performance liquid chromatography system (Waters, Milford, MA) and a Micromass Quattro Ultima mass spectrometer (Waters). The analyte and internal standard (verapamil) were separated on a Unison UC-C18 column (5-μm particle size, 2.0 × 30 mm; Intakt, Kyoto, Japan) at a flow rate of 1.0 mL/min using a mobile phase consisting of 0.01 M ammonium acetate and acetonitrile. The gradient conditions for elution were: 10–90% acetonitrile (0.00–1.50 min); 10% acetonitrile (1.50–2.30 min). Using the positive electrospray mode, molecular ions were formed at a capillary voltage of 3.5 kV and a source temperature of 120°C. Cone energies were 45 and 55 V for 4-OH APZ and verapamil, respectively. Mass transition and collision energy of 4-OH APZ was \( m/z \) 325.0→307.2 and 16 eV, and of verapamil was \( m/z \) 455.4→165.0 and 34 eV, respectively, in multiple reaction monitoring.

**Determination of metabolite concentrations in incubation mixtures with P450 enzymes.** The concentrations of 1'-OH APZ, 4-OH APZ, 1'-OH MDZ, and 4-OH MDZ in the incubation mixtures of liver (intestine) microsomes and recombinant P450 enzymes were determined using an ACQUITY ultra performance liquid chromatography system (Waters) and a Xevo triple quadrupole mass spectrometer (Waters). The analytes and internal standard (MDZ) were separated on an ACQUITY UPLC BEHC18 column (1.7-μm particle size, 2.1 × 30 mm; Waters) at a flow rate of 0.5 mL/min using a mobile phase consisting of 0.01 M ammonium acetate (A) and 0.01 M ammonium acetate/acetonitrile (1:9, v/v) (B). The gradient conditions for elution were: 10–100% B (0.00–0.20 min); 100% B (0.40–0.70 min); 100–10% B (0.70–0.71 min); and 10% B (0.71–1.00 min). Using the positive electrospray mode, molecular ions
were formed at a capillary voltage of 0.5 kV and a source temperature of 150°C. Mass transition and collision energy of 1'-OH APZ was \( m/z \) 325.1→297.1 and 25 eV, 4-OH APZ was \( m/z \) 325.1→280.2 and 25 eV, 1'-OH MDZ was \( m/z \) 341.9→203.3 and 30 eV, 4-OH MDZ was \( m/z \) 341.9→325.3 and 20 eV, APZ (internal standard) was \( m/z \) 309.1→76.8 and 35 eV, and MDZ (internal standard) was \( m/z \) 326.1→291.1 and 25 eV, respectively, in multiple reaction monitoring.

\textit{Determination of APZ and RIF concentrations in plasma samples.} Methanol (100 µL) containing 100 µg/mL ascorbic acid and 20 µL internal standard solution (methanol containing 100 µg/mL APZ-d5) were added to 100-µL aliquots of plasma samples. After the addition of 80 µL acetonitrile, the mixtures were centrifuged and aliquots of supernatants were analyzed using an LC20A chromatography system (Shimadzu, Kyoto, Japan) and an API 4000 mass spectrometer (Sciex Division of MDS, Toronto, Ontario, Canada). Reversed-phase chromatographic separation was achieved on a Cadenza CD-C18 column (4.6 × 50 mm, 3-µm particle size; Imtakt, Kyoto, Japan). The mobile phase consisted of A, 0.01 M ammonium acetate (pH 4.0) and B, acetonitrile in isocratic conditions at 60% A and 40% B. The flow rate was 1.0 mL/min and the column was kept at 35°C. Data were acquired in the positive ion mode with atmospheric pressure chemical ionization. The following multiple reaction monitoring transitions were used for the detection of analytes: 309→205 \( m/z \) for APZ, 314→210 \( m/z \) for APZ-d5, and 823→399 \( m/z \) for RIF.

\textit{Data Analysis.} The concentrations of KTZ producing 50% decrease in APZ 4- and 1'-hydroxylase activities (IC\textsubscript{50}) were determined by nonlinear regression analysis using Prism software (version 5.01; GraphPad Software Inc., San Diego, CA). In recombinant systems, APZ showed sigmoidal kinetics, as
reported previously (Galetin et al., 2004). Therefore, untransformed data on metabolic rates were fitted to the 2-site model used in this study by nonlinear regression analysis using Prism software, and kinetic parameters $V_{\text{max}}$, $K_s$ (substrate dissociation constant), and $\alpha$ (defining changes in binding affinity) were calculated. The $CL_{\text{max}}$, the maximum clearance when the enzyme is fully activated, was calculated by the following equation:

$$CL_{\text{max}} = \frac{V_{\text{max}}}{K_S} \times \frac{\sqrt{1/\alpha} - 1}{2(1-\alpha)}$$

To estimate the concentration of RIF producing a 50% increase in APZ 4-hydroxylase activity ($EC_{50}$), concentration-response curves were fitted to the following equation by nonlinear regression analysis using Prism software:

$$E = \frac{E_{\text{max}} \cdot C}{C + EC_{50}}$$

where $E$ and $E_{\text{max}}$ are the effects and maximal effects (expressed as -fold over control), respectively, measured in the presence of RIF (at concentration C).

Peak plasma concentrations ($C_{\text{max}}$) and the time to reach peak plasma concentrations ($T_{\text{max}}$) were obtained directly by observation. The terminal elimination half-life ($t_{1/2}$) was calculated by the relationship $t_{1/2} = 0.693/\lambda$. The area under the plasma concentration-time profile (AUC) was calculated using the trapezoidal rule up to the last measurable concentration, and the AUC was extrapolated to infinity using the $\lambda$ value ($AUC_{\text{inf}}$). Total plasma clearance ($CL_{\text{tot}}$) was calculated as the intravenous dose divided by $AUC_{\text{inf}}$. For intravenous dosing, the steady-state volume of distribution ($V_{ss}$) was calculated as $V_{ss} = \text{MRT} \cdot CL_{\text{tot}}$ with the mean residence time (MRT) defined as the area under the first moment of the plasma
concentration-time profile divided by AUC\textsubscript{inf}. The bioavailability ($F$) was determined from AUC\textsubscript{inf} following oral and intravenous administration.

Because less than 1% of the intravenous dose of APZ was excreted unchanged in urine (data not shown), hepatic blood clearance was assumed to be equal to total blood clearance. The hepatic extraction ratio ($E_H$) and hepatic availability ($F_H$) were estimated by the following equations,

$$E_H = \frac{CL_{tot}}{R_B \cdot Q_H}$$

$$F_H = 1 - E_H$$

where $R_B$ is the blood-to-plasma concentration ratio which was determined \textit{in vitro} to be approximately unity, and $Q_H$ is the hepatic blood flow rate (45 mL/min/kg) (Davies and Morris, 1993).

Oral bioavailability is a function of $F_A$, $F_G$, and $F_H$, as given by the following equation,

$$F = F_A \cdot F_G \cdot F_H$$

where $F_A$ and $F_G$ refer to the fractions of the dose absorbed from the gut lumen and not metabolized by intestinal metabolic enzymes, respectively. The product of $F_A \cdot F_G$ is estimated as follows:

$$F_A \cdot F_G = \frac{F}{F_H}$$

Based on a report that oral bioavailability of APZ is 80–100% in humans (Greenblatt and Wright, 1993), absorption of APZ from the monkey gut lumen was assumed to be complete ($F_A = 1$). Thus, the intestinal extraction ratio ($E_G$) was calculated as follows:

$$E_G = 1 - \frac{F}{F_H}$$

The effects of RIF treatment were compared between pharmacokinetic parameters obtained in the...
control situation (before RIF treatment) with those obtained after RIF treatment by one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparison test, using Prism software. A $P$ value of less than 0.05 was considered statistically significant.
Results

**APZ Metabolism in Liver and Small Intestine Microsomes.** APZ 1'- and 4-hydroxylation activities in liver and small intestine microsomes are shown in Table 1. Higher activities in both liver and intestine microsomes were observed in monkeys as compared to humans. Human and monkey liver microsomes exhibited higher rates of 4-hydroxylation than those of 1'-hydroxylation, which is in agreement with previous observations (von Moltke et al., 1993). Similarly, higher rates of 4-hydroxylation compared with 1'-hydroxylation were observed in human and monkey intestinal microsomes.

MDZ 1'- and 4-hydroxylation activities were also measured in liver and intestine microsomes of humans and monkeys (Table 1). 1'-Hydroxylation was the dominant pathway in the liver and intestine in both species. In human liver and intestine microsomes, MDZ 1'-hydroxylation activities were 37- and 33-fold higher, respectively, than APZ 4-hydroxylation activities. Similarly, in monkeys, hepatic and intestinal microsomal activities of 1'-OH MDZ formation were 32- and 24-fold higher, respectively, than those of 4-OH APZ formation.

KTZ, a CYP3A-specific inhibitor, inhibited APZ 1'- and 4-hydroxylation in human liver and intestine microsomes in a concentration-dependent manner, and the values of IC$_{50}$ (Table 2) were comparable to those reported previously (von Moltke et al., 1993). Inhibition by KTZ was also observed in monkey liver and intestine microsomes in which IC$_{50}$ values were comparable to those obtained with respective human samples (Table 2). These results indicate that CYP3A-mediated metabolism is the predominant elimination pathway of APZ in monkeys as well as in humans.
APZ Metabolism with Recombinant P450 Isoforms. We measured the formation rates of 4-OH and 1'-OH APZ over a range of concentrations (2.5–1000 μM) of APZ using recombinant human and cynomolgus monkey CYP3A enzymes. Human CYP3A4 showed a higher 4-hydroxylase activity as compared with human CYP3A5 (Fig. 1A). Similarly, cynomolgus CYP3A4 exhibited higher 4-hydroxylase activity than cynomolgus CYP3A5 (Fig. 1B). Cynomolgus CYP3A4 and CYP3A5 showed higher metabolic rates per molecule of P450 than human CYP3A4 and CYP3A5, respectively, for 4-hydroxylation, the major metabolic pathway of APZ in the 2 species (Table 3). The 1'-hydroxylation rates were higher for human CYP3A5 as compared with human CYP3A4 (Fig. 1C), which is consistent with previous findings obtained using baculovirus-expressed recombinant CYP3A4 and CYP3A5 (Galetin et al., 2004). In contrast, cynomolgus CYP3A5 showed higher 1'-hydroxylase activity than cynomolgus CYP3A4 (Fig. 1D). APZ showed sigmoidal kinetics in the examined concentration ranges, and Eadie-Hofstee plots clearly showed curvature for the 2 oxidation reactions, regardless of the tested isoforms (Fig. 2, Table 3).

von Moltke et al. (1993) reported Michaelis constants (K_m) of 170–305 and 221 μM for APZ 4-hydroxylation in human and monkey liver microsomes, respectively. Gorski et al. (1999) reported K_m values of 516–963 μM for APZ 4-hydroxylation in human liver microsomes. The values were comparable to our estimates of K_s and/or α·K_s for the 4-hydroxylation by recombinant CYP3A4 (the predominant isoform) as shown in Table 3.

In Vitro Induction of APZ 4-Hydroxylase Activity by RIF. To validate the use of APZ for in vivo
induction study in monkeys, we examined the effect of RIF on APZ 4-hydroxylase activity in primary cultures of cryopreserved hepatocytes of 2 cynomolgus monkeys. RIF increased APZ 4-hydroxylase activity in a concentration-dependent manner (Fig. 3). The EC$_{50}$ values for the 2 hepatocyte preparations were 2.4 μM (95% confidence interval, 1.1–5.2 μM) and 1.7 μM (95% confidence interval, 1.0–3.0 μM). These results were comparable with the previously reported EC$_{50}$ values (0.67–2.34) for the induction of MDZ 1'-hydroxylase activity by RIF in cynomolgus monkey hepatocytes (Kim et al., 2009).

**Effects of RIF on Pharmacokinetics of APZ in Monkeys.** The pharmacokinetic parameters of APZ were determined in cynomolgus monkeys after intravenous and oral administration at 0.5 mg/kg (Fig. 4; Tables 4 and 5). RIF treatment resulted in significant and maximal changes in pharmacokinetic parameters of APZ on the last day of the treatment or on a day later. The CL$_{tot}$ showed a 3.3- to 4.1-fold increase, which was a more pronounced effect than that on V$_{dss}$. The reasons for changes observed in V$_{dss}$ after RIF treatment are unclear. AUC after oral administration was decreased by 85–90%. The t$_{1/2}$ following oral and intravenous dosing was reduced. Plasma RIF concentrations after the last dose (5$^{th}$ dosing day) were maintained above 5 μM for a period of 6 h (Fig. 5). Even 1 week after the last dose of RIF, AUC following intravenous dosing was significantly decreased by 66% compared with that before RIF treatment. AUC of oral APZ was also decreased by 78% 1 week after RIF withdrawal, although the change was not statistically significant. The pharmacokinetic parameters of APZ returned to near baseline levels 4 weeks after discontinuing RIF treatment.

The oral dose of APZ was assumed to be completely absorbed from the gut wall in monkeys (F$_A$ = 1) as
observed in humans. RIF is known to induce efflux transporters such as P-glycoprotein (P-gp) in the intestine, and also has been shown to inhibit uptake transporters such as organic anion transporting polypeptides (OATPs). To date, there has been no report on whether APZ is a substrate for such drug transporters. However, MDZ, a closely structurally related benzodiazepine, has been shown not to be actively transported by P-gp or OATPs \textit{in vitro} (Gombar et al., 2004; Franke et al., 2008). In addition, it is unlikely that intestinal membrane transporters may have any significant impact on the fraction absorbed for APZ under our experimental conditions, because of its high solubility and high permeability. Therefore, we evaluated the changes in $E_H$ and $E_G$ values assuming that the $F_A$ value of APZ was not affected by RIF treatment.

Estimates for first-pass hepatic ($E_H$) and intestinal ($E_G$) extraction ratios after oral administration of APZ are shown in Fig. 6. $E_H$ was increased from $0.09 \pm 0.03$ before RIF treatment to $0.31 \pm 0.03 \ (P < 0.05)$ on the day of the last RIF dose and to $0.39 \pm 0.04 \ (P < 0.05)$ a day later. Although not statistically significant, an increase in $E_G$ from $0.45 \pm 0.06$ in the pre-induction phase to $0.63 \pm 0.16$ on the last day of RIF treatment was also observed.

We observed that the value for $E_G \ (0.24 \pm 0.19)$ 11 weeks after RIF treatment was fairly low compared with that at the pre-induction phase ($0.45 \pm 0.06$). This is probably a consequence of intra-individual variability in one of the three animals. At Week 11, the AUC value for the animal was 23% lower in the i.v. study and 30% higher in the p.o. study than those before the RIF treatment. These two data, which gave a higher $E_H$ value and a higher $F$ (bioavailability) value, cooperatively made an estimate of $E_G$ lower
than that in the pre-induction phase. The other two animals showed lower intra-individual variability.
Discussion

DDI related to enzyme induction in the liver and intestine is a major concern in drug development. One useful preclinical approach for assessing the induction potential of drug candidates is to establish an \textit{in vivo} animal model which allows for the evaluation of effects on pharmacokinetics of a specific substrate for drug-metabolizing enzymes of interest. In the present study, we evaluated changes in both prehepatic and hepatic drug clearance in cynomolgus monkeys produced by a representative inducer of CYP3A.

Monkeys tend to exhibit a lower oral bioavailability than humans, despite similarities in functional activities and amino acid sequences of drug-metabolizing enzymes between the 2 species (Chiou and Buehler, 2002). A recent study suggested that species differences could be mostly attributed not only to hepatic first-pass metabolism but also to the intestinal absorption process (Komura and Iwaki, 2008; Takahashi et al., 2009; Akabane et al., 2010). For example, minimal oral bioavailability of MDZ in cynomolgus monkeys (2–7\%) is not fully explained by hepatic availability (~70\%), but is rather attributed to its low intestinal availability (~10\%) (Kanazu et al., 2004; Sakuda et al., 2006; Ogasawara et al., 2007; Nishimura et al., 2007b). Thus, CYP3A substrates, such as MDZ, showing a high clearance in monkeys are not suitable as \textit{in vivo} markers of enzyme induction, because drug-induced up-regulation of CYP3A expression is unlikely to result in any detectable increase in first-pass metabolism of substrates, particularly in the intestine. In addition, pharmacokinetics of high-clearance drugs is much less sensitive to changes in enzyme activity when drugs are administered intravenously. Prueksaritanont et al. (2006) reported that RIF did not significantly affect the i.v. kinetics of MDZ in monkeys.
In the present study, we selected APZ as a marker substrate, which has been shown to be a useful probe for human CYP3A activity \textit{in vivo} (Schmider et al., 1999) and \textit{in vitro} (von Moltke et al., 1993; Schmider et al., 1996). Small intestinal microsomes, similar to liver microsomes, catalyzed APZ 1′- and 4-hydroxylation in monkeys as well as in humans (Table 1). KTZ, a potent CYP3A inhibitor, exhibited extremely low IC\textsubscript{50} values for microsomal oxidative reactions in monkeys, as observed in humans (Table 2).

In addition, the urinary excretion rate of unchanged drug was less than 1% of the dose after intravenous administration of APZ to monkeys (data not shown). From these data, we conclude that CYP3A-mediated oxidative metabolism is predominantly involved in the elimination of APZ in monkeys.

\textit{In vitro} studies using recombinant enzymes showed that APZ 4-hydroxylation, the major metabolic pathway in both humans and monkeys, is catalyzed predominantly by CYP3A4 (Fig. 1). Furthermore, APZ showed sigmoidal kinetics in recombinant systems for both species, indicating that CYP3A enzymes exhibited similar kinetic properties for APZ between humans and monkeys (Fig. 2, Table 3). Based on these findings, we selected APZ as an alternative marker of CYP3A activity in monkeys. Our \textit{in vitro} study also revealed that unlike humans, CYP3A4 rather than CYP3A5 showed higher 1′-hydroxylase activity in monkeys, although 1′-hydroxylation is the minor metabolic pathway in both species. In addition, rates per molecule of P450 for APZ 4-hydroxylation were much higher for monkeys than for humans. This finding was not affected by the addition of cytochrome \textit{b}\textsubscript{5} in the recombinant systems (data not shown). This is apparently consistent with the \textit{in vivo} observation that the oral bioavailability of APZ was lower in monkeys (~50%) compared with that in humans (80–100%) (Greenblatt and Wright, 1993).
However, species differences in the recombinant systems might not necessarily be extrapolated to the in vivo situation, and it remains unclear whether expression levels of CYP3A enzymes in monkeys are higher than those in humans. Thus, further studies are necessary to investigate the significance of species differences in recombinant enzyme activity.

Monkey liver and intestine microsomes showed lower metabolic activity for APZ than for MDZ (Table 1). Additional studies using recombinant P450 enzymes supported remarkable differences in the metabolic activity between substrates (data not shown). This may account for the moderate bioavailability of APZ in monkeys (50%), whereas several other CYP3A substrates undergo extensive first-pass metabolism (Takahashi et al., 2009). RIF treatment significantly increased $CL_{tot}$ and $E_{H}$ values of APZ (Table 4 and Fig. 6), indicating hepatic CYP3A induction. It can be concluded that APZ is a moderate-clearance drug and thereby a sensitive in vivo marker for CYP3A induction in monkeys. RIF also tended to increase $E_{G}$ values, although the changes were not statistically significant.

In the present study, we examined the pharmacokinetics of APZ for up to 11 weeks after discontinuing RIF treatment to investigate the time course of changes in CYP3A activity. A similar study was reported in humans given intravenous and oral doses of verapamil, and the half-life of decrease in CYP3A activity after RIF withdrawal was estimated to be 1.5–2.1 days (Fromm et al., 1996). In another clinical study, urinary ratios of 6β-hyrdroxcortisol to cortisol, an endogenous marker for CYP3A activity, returned to pre-induction levels 11 days after stopping RIF treatment (Tran et al., 1999), and the mean half-life of the return from maximum to basal levels was estimated to be 3 days (Yang et al., 2008). Compared with these
estimates, the rate of recovery of CYP3A enzyme in monkeys seems to be slower because AUC of APZ following intravenous and oral administration was decreased by 66% and 78%, respectively, even 1 week after RIF withdrawal. The reasons for the discrepancy are unclear, but one possibility is that the turnover of CYP3A enzymes may be lower in monkeys than humans. It is unlikely that hepatic and/or intestinal exposures to RIF in monkeys could account for any persistent inductive effect, because plasma concentrations of RIF 24 h after the last dose decreased below the quantification limit (approximately 0.06 μM) and to the level at which RIF did not affect APZ 4-hydroxylase activity in primary cultured hepatocytes (Fig. 3).

It is a common practice to use human tissue preparations in vitro to investigate the potential of new chemical entities to cause enzyme induction during the early stages of drug discovery. Prediction models have been proposed that incorporate in vitro induction data into mathematical equations to predict changes in clearance for CYP3A substrates (Fahmi et al., 2008; Shou et al., 2008; Kozawa et al., 2009). However, these models have limitations. First, an empirical aspect is associated with mathematical equations where a scaling factor is included to optimize in vitro to in vivo extrapolation. Second, some of the models do not address changes in the inducer concentration with time or do not consider intestinal enzyme induction. We propose an alternative method that overcomes these limitations. Our animal model allows assessing the extent of prehepatic and hepatic induction in relation to the time course of exposure to potential inducers. Together with clinical pharmacokinetic data, findings obtained from the proposed preclinical DDI study may provide guidance for clinical DDI study design.
As an inducer we used RIF, a representative ligand of the pregnane X receptor (PXR), which plays a major role in drug-induced activation of CYP3A expression in human liver and small intestine. The nucleotide sequences of the ligand-binding domains between rhesus monkey and human PXR are 96% identical (Moore et al., 2002). *In vitro* studies using rhesus monkey hepatocytes showed that RIF induced CYP3A4 mRNA with an EC$_{50}$ value comparable to that of CYP3A4 mRNA induction in human hepatocytes (Faucette et al., 2004; Prueksaritanont et al., 2006). Our results from cynomolgus monkey hepatocytes were substantially consistent with those from rhesus monkey hepatocytes (Fig. 3). Pretreatment with RIF reduced AUC of APZ after oral administration to similar extents as observed in a previous clinical DDI study (decreased by 88%, Schmider et al., 1999) and our current investigation (decreased by 85–90%; Fig. 4B, Table 5). In both cases, RIF concentrations in plasma exceeded those shown to achieve maximum CYP3A induction effects in cultured hepatocytes (Prueksaritanont et al., 2006; Nishimura et al., 2007a; Fig. 5). However, concentration-response relationships could vary among inducers, nuclear receptors, or the species examined. Kim et al. (2009) investigated PXR transactivation profiles of 30 compounds, including known CYP3A4 inducers, in reporter assays, demonstrating that EC$_{50}$ values correlated well between cynomolgus and human PXRs, although there were notable species differences in EC$_{50}$ and E$_{max}$ values for reserpine. Thus, further studies using other inducers are required for a better understanding of similarities and differences in CYP3A induction *in vivo* between humans and monkeys.

In conclusion, APZ is a more sensitive marker to evaluate CYP3A induction in monkeys, compared
with other CYP3A substrates showing higher clearance. The pharmacokinetic changes of APZ in RIF-treated monkeys were comparable to previous reports on clinical DDIs involving CYP3A induction. Thus, the potential for drug candidates to cause clinically relevant CYP3A induction can be assessed preclinically by investigating the effect on pharmacokinetics of APZ in monkeys.
Acknowledgements

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References


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Legends for figures.

Fig. 1 Rates of 4-OH (A and B) and 1'-OH (C and D) APZ formation by recombinant CYP3A enzymes form human and cynomolgus monkey. Metabolic rates were compared between human CYP3A4 (●) and CYP3A5(○) in A and C, and between cynomolgus CYP3A4 (▲) and CYP3A5 (△) in B and D. Data points represent duplicate determinations.

Fig. 2 Eadie-Hofstee plots for APZ 4-hydroxylase activity by recombinant human CYP3A4 (A) and cynomolgus CYP3A4 (B). Data points represent duplicate determinations.

Fig. 3 Effects of RIF on APZ 4-hydroxylase activity in cynomolgus monkey hepatocytes. Results from two hepatocyte preparations (open and closed bars) are shown as the mean of duplicate determinations.

Fig. 4 Effects of once-daily RIF pretreatment (20 mg/kg, p.o. for 5 days) on the plasma concentration time profiles of APZ after intravenous (A) and oral (B) administration to cynomolgus monkeys at a dose of 0.5 mg/kg. APZ was administered one week before (open circles), on the last day of (solid circles), one day after (solid triangles), one week after (solid squares), 4 week after (open squares), and 11 week after (open triangles) the RIF pretreatment. Each point represents the mean ± S.D. of data from three monkeys.

Fig. 5 Plasma concentration time profiles of RIF after the last dose of once-daily administration (20
mg/kg, p.o. for 5 days) to cynomolgus monkeys. Each point represents the mean ± S.D. of data from three
monkeys.

Fig. 6 Effects of RIF pretreatment on the hepatic (open bars) and intestinal (solid bars) extraction ratio of
APZ after administration to cynomolgus monkeys. Each value represents the mean ± S.D. of data from
three monkeys. *: P < 0.05 compared with control (Pre-RIF)
### Table 1

*Activities for APZ and MDZ metabolite formation in liver and small intestine microsomes from humans and cynomolgus monkeys*

Activity was measured at a substrate concentration of 10 μM. Each value is represented as the mean of duplicate incubations.

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>Liver microsomes</th>
<th>Intestinal microsomes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Human</td>
<td>Monkey</td>
</tr>
<tr>
<td>1′-OH APZ</td>
<td>0.0062</td>
<td>0.0094</td>
</tr>
<tr>
<td>4-OH APZ</td>
<td>0.030</td>
<td>0.097</td>
</tr>
<tr>
<td>1′-OH MDZ</td>
<td>1.1</td>
<td>3.1</td>
</tr>
<tr>
<td>4-OH MDZ</td>
<td>0.10</td>
<td>1.0</td>
</tr>
</tbody>
</table>
Table 2

Inhibition of KTZ on APZ metabolite formation in liver and small intestine microsomes from humans and cynomolgus monkeys

Activity was measured at a substrate concentration of 100 μM. IC₅₀ values are calculated for duplicate incubations.

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>Liver microsomes</th>
<th>Intestinal microsomes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Human</td>
<td>Monkey</td>
</tr>
<tr>
<td>IC₅₀ (μM)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1'-OH APZ</td>
<td>0.071</td>
<td>0.086</td>
</tr>
<tr>
<td>4-OH APZ</td>
<td>0.035</td>
<td>0.069</td>
</tr>
</tbody>
</table>
Table 3

*Kinetic parameters for APZ metabolite formation in recombinant human and cynomolgus monkey CYP3A enzymes expressed in E. coli (mean ± S.E.)*

<table>
<thead>
<tr>
<th>Species</th>
<th>Pathway</th>
<th>Enzyme</th>
<th>$V_{\text{max}}$</th>
<th>$K_s$</th>
<th>$\alpha$</th>
<th>$CL_{\text{max}}$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>pmol/min/pmol P450</td>
<td>$\mu$M</td>
<td></td>
<td>$\mu$L/min/pmol P450</td>
</tr>
<tr>
<td>Human</td>
<td>1'-Hydroxy</td>
<td>CYP3A4</td>
<td>0.47 ± 0.02</td>
<td>390 ± 15</td>
<td>0.32 ± 0.04</td>
<td>1.29</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CYP3A5</td>
<td>2.22 ± 0.11</td>
<td>1400 ± 93</td>
<td>0.020 ± 0.004</td>
<td>5.65</td>
</tr>
<tr>
<td></td>
<td>4-Hydroxy</td>
<td>CYP3A4</td>
<td>11.99 ± 0.44</td>
<td>652 ± 24</td>
<td>0.13 ± 0.01</td>
<td>27.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CYP3A5</td>
<td>3.09 ± 0.16</td>
<td>1367 ± 90</td>
<td>0.025 ± 0.004</td>
<td>7.29</td>
</tr>
<tr>
<td>Monkey</td>
<td>1'-Hydroxy</td>
<td>CYP3A4</td>
<td>1.17 ± 0.06</td>
<td>215 ± 14</td>
<td>0.26 ± 0.07</td>
<td>6.18</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CYP3A5</td>
<td>0.71 ± 0.04</td>
<td>179 ± 12</td>
<td>0.30 ± 0.08</td>
<td>4.32</td>
</tr>
<tr>
<td></td>
<td>4-Hydroxy</td>
<td>CYP3A4</td>
<td>87.78 ± 4.52</td>
<td>296 ± 17</td>
<td>0.25 ± 0.05</td>
<td>342</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CYP3A5</td>
<td>15.71 ± 0.88</td>
<td>242 ± 16</td>
<td>0.18 ± 0.04</td>
<td>83.5</td>
</tr>
</tbody>
</table>
Table 4

Pharmacokinetic parameters of APZ after a single intravenous administration (0.5 mg/kg) to cynomolgus monkeys pretreated with vehicle or RIF (20 mg/kg) for 5 days

Each value represents the mean ± S.D. of three monkeys.

<table>
<thead>
<tr>
<th></th>
<th>Before RIF treatment</th>
<th>After RIF treatment</th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Day of the last RIF dosing</td>
<td>One day after the last RIF dosing</td>
<td>One week after the last RIF dosing</td>
<td>4 weeks after the last RIF dosing</td>
<td>11 weeks after the last RIF dosing</td>
<td></td>
</tr>
<tr>
<td>AUC$_\text{inf}$ (ng·h/mL)</td>
<td>2131 ± 814</td>
<td>606 ± 56*</td>
<td>480 ± 40*</td>
<td>722 ± 152*</td>
<td>1509 ± 716</td>
<td>1762 ± 527</td>
<td></td>
</tr>
<tr>
<td>CL$_\text{tot}$ (mL/h/kg)</td>
<td>255 ± 82</td>
<td>830 ± 80*</td>
<td>1048 ± 91*</td>
<td>713 ± 147*</td>
<td>377 ± 145</td>
<td>299 ± 77</td>
<td></td>
</tr>
<tr>
<td>V$_\text{ss}$ (mL/kg)</td>
<td>1110 ± 100</td>
<td>1391 ± 127</td>
<td>1500 ± 108*</td>
<td>1476 ± 192*</td>
<td>1371 ± 143</td>
<td>1157 ± 49</td>
<td></td>
</tr>
<tr>
<td>t$_{1/2}$ (h)</td>
<td>3.7 ± 1.2</td>
<td>1.1 ± 0.2*</td>
<td>1.0 ± 0.1*</td>
<td>1.8 ± 0.9</td>
<td>3.4 ± 1.0</td>
<td>3.2 ± 1.0</td>
<td></td>
</tr>
</tbody>
</table>

* Significantly different compared with the control values obtained before RIF treatment ($P < 0.05$).
Table 5

Pharmacokinetic parameters of APZ after a single oral administration (0.5 mg/kg) to cynomolgus monkeys pretreated with vehicle or RIF (20 mg/kg) for 5 days

Each value represents the mean ± S.D. of three monkeys.

<table>
<thead>
<tr>
<th></th>
<th>Before RIF treatment</th>
<th>Day of the last RIF dosing</th>
<th>One day after the last RIF dosing</th>
<th>One week after the last RIF dosing</th>
<th>4 weeks after the last RIF dosing</th>
<th>11 weeks after the last RIF dosing</th>
</tr>
</thead>
<tbody>
<tr>
<td>$C_{\text{max}}$ (ng/mL)</td>
<td>92.3 ± 44.7</td>
<td>41.0 ± 20.5</td>
<td>40.4 ± 21.6</td>
<td>55.8 ± 23.7</td>
<td>101.2 ± 29.6</td>
<td>124.6 ± 63.8</td>
</tr>
<tr>
<td>$T_{\text{max}}$ (h)</td>
<td>4.7 ± 1.2</td>
<td>2.7 ± 1.2</td>
<td>1.7 ± 0.6*</td>
<td>2.7 ± 1.2</td>
<td>4.0 ± 0.0</td>
<td>4.7 ± 1.2</td>
</tr>
<tr>
<td>AUC_{inf} (ng-h/mL)</td>
<td>1078 ± 468</td>
<td>159 ± 83</td>
<td>113 ± 72*</td>
<td>242 ± 149</td>
<td>775 ± 389</td>
<td>1258 ± 738</td>
</tr>
<tr>
<td>$F$ (%)</td>
<td>50 ± 6</td>
<td>26 ± 12</td>
<td>23 ± 13</td>
<td>32 ± 14</td>
<td>51 ± 7</td>
<td>68 ± 19</td>
</tr>
<tr>
<td>$t_{1/2}$ (h)</td>
<td>5.6 ± 2.4</td>
<td>1.1 ± 0.2*</td>
<td>1.1 ± 0.2*</td>
<td>1.7 ± 1.0*</td>
<td>3.3 ± 1.0</td>
<td>3.9 ± 1.2</td>
</tr>
</tbody>
</table>

* Significantly different compared with the control values obtained before RIF treatment ($P < 0.05$).
Figure 1

A. 4-Hydroxy alprazolam formation (pmol/min/pmol P450) vs. Alprazolam concentration (μM)

B. 4-Hydroxy alprazolam formation (pmol/min/pmol P450) vs. Alprazolam concentration (μM)

C. 1'-Hydroxy alprazolam formation (pmol/min/pmol P450) vs. Alprazolam concentration (μM)

D. 1'-Hydroxy alprazolam formation (pmol/min/pmol P450) vs. Alprazolam concentration (μM)