

DMD#9878

***IN VITRO* AND *IN VIVO* CYP3A64 INDUCTION AND INHIBITION
STUDIES IN RHESUS MONKEYS:**

A preclinical approach for CYP3A-mediated drug interaction studies

THOMAYANT PRUEKSARITANONT, YUHSIN KUO, CUYUE TANG, CHUNZE
LI, YUE QIU, BING LU, KRISTIE STRONG-BASALYGA, KAREN RICHARDS,
BRIAN CARR, JIUNN H. LIN

Department of Drug Metabolism, Merck Research Laboratories, West Point, PA

DMD#9878

Running Title: CYP3A induction and inhibition studies in rhesus monkeys

Corresponding author:

Thomayant Prueksaritanont, Ph.D.

Department of Drug Metabolism

WP75-100

Merck Research Laboratories

West Point, PA 19486

Telephone: 215-652-4194.

E-mail: thomayant_prueksaritanont@merck.com

Number of:

Text pages – 28

Tables – 3

Figures – 7

References – 40

Words in:

- abstract – 266
- introduction – 708
- discussion – 1584

ABBREVIATIONS: MDZ, midazolam; 1'-OH MDZ, 1'-hydroxy midazolam; 4-OH MDZ, 4-hydroxy midazolam; AUC, area under plasma concentration-time curve; C_{max} , peak plasma concentration; CL, plasma clearance; F_h , hepatic availability; V_{dss} , volume of distribution at steady-state; $t_{1/2}$, half-life; i.p.v., intra-hepatic portal vein; IS, internal standard; LC-MS/MS, liquid chromatography coupled with tandem mass spectrometry.

DMD#9878

ABSTRACT

In this study, induction and inhibition of rhesus monkey CYP3A64 versus human CYP3A4 were characterized *in vitro*, and the corresponding pharmacokinetic consequences were evaluated in rhesus monkeys. In monkey hepatocytes, rifampin markedly induced CYP3A64 mRNA ($EC_{50} = 0.5 \mu\text{M}$; $E_{\text{max}} = 6\text{-fold}$) and midazolam (MDZ) 1'-hydroxylase activity ($EC_{50} = 0.2 \mu\text{M}$; $E_{\text{max}} = 2\text{-fold}$). Compound A, a known potent and mechanism-based inhibitor of CYP3A4, strongly inhibited the formation of 1'-hydroxy MDZ by recombinant CYP3A64 in a concentration- and time-dependent manner ($K_i = 0.25 \mu\text{M}$; $k_{\text{inact}} = 0.4 \text{ min}^{-1}$). Similar corresponding results also were obtained with human CYP3A4 in the presence of rifampin or compound A. In rhesus monkeys, MDZ exhibited a relatively high metabolic clearance (primarily via 1'-hydroxylation followed by glucuronidation), and a low hepatic availability ($F_h = 16\%$). Consistent with the induction of hepatic metabolism of a high clearance compound, pretreatment with rifampin (18 mg/kg p.o. for 5 days) did not significantly affect the i.v. kinetics of MDZ, but caused a pronounced reduction (~10-fold) in the systemic exposure to MDZ and consequently its F_h following intra-hepatic portal vein (i.pv.) administration of MDZ. A comparable extent of the pharmacokinetic interaction also was obtained after a 1.8 mg/kg rifampin dose. Also consistent with the *in vitro* CYP3A64 inhibition finding, compound A (6 mg/kg i.v.) markedly increased (10-fold) the i.pv. administered MDZ exposure. At the doses studied, plasma concentrations of rifampin or compound A reached or exceeded their respective *in vitro* EC_{50} or K_i values. These findings suggest the potential applicability of the *in vitro* – *in vivo* relationship approach in rhesus monkeys for studying CYP3A-mediated interactions in humans.

Introduction

Many severe adverse drug responses following multiple drug therapies have been reported to be associated with pharmacokinetic drug interactions (Lesar et al., 1997; Dresser et al., 2000). Inhibition of drug metabolizing enzymes, primarily CYP3A, is the main reason for drug interactions reported in the literature (Thummel and Wilkinson, 1998; Dresser et al., 2000). Induction of CYP3A, although less common, is also known to cause profound clinical effects, either by reducing therapeutic efficacy of drugs or enhancing toxicity from toxic or reactive metabolites (Thummel and Wilkinson, 1998). CYP3A4 is the most abundant of all of the human isoforms and catalyzes the metabolism of a large number of compounds including clinically important pharmaceuticals. Accordingly, during lead optimization and identification in early drug discovery processes, the potential for new chemical entities to cause CYP3A4 inhibition and induction is usually assessed, primarily *in vitro* using human tissue preparations (Weaver, 2001; Worboys and Carlile, 2001). Such *in vitro* studies are widely accepted to have potential in identifying the likelihood of drug interactions *in vivo* (Tucker et al., 1998; Bjornsson et al., 2003). However, to help in drug candidate selection, there is often a need to prospectively predict, based on the *in vitro* results, the likely extent of drug interactions and subsequent clinical importance. Although there have been several successful quantitative predictions reported for drug interactions mediated via enzyme inhibition, these are mainly retrospective and not applicable to all cases (Houston and Galetin, 2003; Ito et al., 2004). To date, systematic extrapolations of the *in vitro* data to *in vivo* situations have not been extensively attempted for drug interactions mediated via enzyme induction (Bjornsson et al., 2003).

One of the general approaches commonly used to help form or verify a basis for extrapolating *in vitro* human data to clinical outcomes during early drug development involves *in vitro* – *in vivo* assessments in an animal model under conditions reflective of a likely therapeutic scenario (Kanazu et al., 2004). Such an *in vitro* – *in vivo* preclinical model is also highly desirable

DMD#9878

in a later development stage to help provide a mechanistic insight for the interpretation of interactions observed clinically (Prueksaritanont et al., 2002). For metabolic interaction studies, this approach requires that the animal model possesses similarities to humans with respect to enzymatic activities, in the absence and presence of an enzyme inducer or inhibitor of interest, as well as the basic *in vivo* ADME characteristics of a selected probe substrate. In this regard, rhesus monkeys (*Macaca mulatta*) and cynomologous monkeys (*Macaca fascicularis*), non-human primates widely used in the pharmaceutical industry for preclinical evaluations of drug absorption and disposition as well as safety and toxicity, have generally been considered to be more appropriate animal models than rodents for drug metabolism studies, based on the sequence and functional activity similarities of drug metabolizing enzymes (Sharer et al., 1995; Igarashi et al., 1997; Kanazu et al., 2004). Recently, rhesus monkey CYP3A64 has been cloned and characterized *in vitro*, in comparison with human CYP3A4 and members of CYP3A from other preclinical species (Carr et al., manuscript in review). The results showed that CYP3A64 was most similar, in protein sequences and functional activities, to human CYP3A4, suggesting that the rhesus monkey may serve as a valuable preclinical model to assess CYP3A-mediated drug interactions. However, the susceptibility of CYP3A64 to known enzyme inhibitors and inducers, both *in vitro* and *in vivo*, has not been studied.

Thus, to help assess the potential utility of the rhesus monkey as a preclinical model for studying CYP3A-mediated drug interactions, we set out to evaluate 1) the *in vitro* induction and inhibition susceptibility profiles of rhesus monkey CYP3A64, relative to human CYP3A4, 2) the corresponding *in vivo* pharmacokinetic consequences, and 3) the respective *in vitro* – *in vivo* induction and inhibition relationships in rhesus monkeys. Rifampin, a potent inducer of CYP3A4 (Neimi et al., 2003) and compound A (structure shown in Chiba et al., 1995), previously shown to be a potent and selective CYP3A4 inhibitor (Chiba et al., 1995; Lin et al., 1995), were chosen as a model inducer and inhibitor, respectively. Midazolam (MDZ), a well established and highly

DMD#9878

sensitive *in vivo* CYP3A4 probe in humans (Thummel et al., 1994; Bjornsson et al., 2003) was employed as an *in vitro* and *in vivo* CYP3A marker substrate in this study.

Materials and Methods

Materials. MDZ, 1'-hydroxy (1'-OH) and 4-hydroxy (4-OH) MDZ, diazepam and rifampin were obtained from Sigma (St. Louis, MO). Compound A (N-[2(R)-hydroxy-1(S)-indanyl]-5-[2(S)-(1,1-dimethylethylaminocarbonyl)-4-[(furo[2,3-b]pyridin-5-yl)-methyl]piperazin-1-yl]-4(S)-hydroxy-2(R)-phenylmethylpentanamide, Chiba et al., 1995) was synthesized at Merck Research Laboratories (West Point, PA). All other reagents were of analytical or HPLC grade. Human recombinant CYP3A4 and rhesus monkey CYP3A64 generated in-house at Merck Research Labs (West Point, PA) were expressed using Invitrogen's Bac-to-Bac™ expression system (Carr et al., manuscript in review). The expression system utilizes Sf21 insect cells infected with baculoviruses containing CYP3A4 or CYP3A64 and NADPH Oxidoreductase (OR). The expression system contained OR and CYP at a final molar ratio of three (OR:CYP). Fresh human hepatocytes from 6 different donors (Caucasian males and females, age 14 – 69 yrs) were obtained from commercial sources (CellzDirect, Tucson, AZ, In Vitro Technologies, Baltimore, MD, and Tissue Transformation Technologies, Edison, NJ) in 24-well culture plates, while rhesus monkey hepatocytes were prepared in-house according to the method of Moldeús et al. (1978). The basal Hepatocyte Culture Medium (HCM) utilized in our studies was custom made and obtained from JRH Biosciences (Lenexa, KS). It was modified from that described in Dich and Grunnet (1990) by adding 10 ml/L of non-essential amino acids (Invitrogen, Carlsbad, CA), 1X penicillin-streptomycin-glutamine (Invitrogen), 4 g/L sodium bicarbonate (Sigma, St. Louis, MO), 10 ml/L insulin-transferrin-selenium premix (BD Biosciences, San Jose, CA), and 10 nM dexamethasone.

***In Vitro* Induction Studies.** The study was conducted using rhesus monkey and human hepatocytes as previously described (Prueksaritanont et al., 2005). In brief, hepatocyte cultures

DMD#9878

were treated, in triplicate for each treatment, for 2 days with culture media containing rifampin or vehicle control (DMSO, 0.1% v/v). Over the treatment period, dosing media were changed daily, and at the end of the treatment (48 hr), medium was aspirated from wells and plates were either stored at -70°C for later RNA isolation and quantitation by a two-step reverse transcription – polymerase chain reaction (RT-PCR), or replaced with Hank's Balanced Salt Solution (HBSS) supplemented with 10 mM HEPES (4-(2-Hydroxyethyl)-1-piperazineethane-sulfonic acid) buffer and returned to the incubator for 10 min, after which the buffer was aspirated and replaced with 10 mM HEPES buffer containing enzyme marker substrates for measuring enzyme activities (see below).

Total RNA was isolated utilizing the RNeasy kit (QIAGEN, Valencia, CA) according to manufacturer's protocol, and quantitated using Quant-iT RiboGreen RNA reagent kit (Molecular Probes, Eugene, OR). The yield was normal and the quality of RNA was indicated by polymerase chain reaction (PCR) results obtained. A two-step reverse transcription – polymerase chain reaction (RT-PCR) was conducted by reverse transcribing 50 ng of total RNA to cDNA using TaqMan® Reverse Transcription Reagents with random hexamer primers, as previously described (Prueksaritanont et al., 2005). Taqman® primers and probes were designed using Primer Express Software v1.0 (PE Applied Biosystems, Foster City, CA) and Genbank accession numbers M18907 and AY334551 for CYP3A4 and CYP3A64, respectively. The primer sequences for CYP3A4 TaqMan set were: Forward – 5'

GTGTGTTTCCAAGAGAAGTTACAAATTT-3'; Reverse – 5'-

CCACTCGGTGCTTTTGTGTATC-3'; and Probe – 5'-FAM-

CGAGGCGACTTTCTTTCATCCTTTTACAGA-TAM-3'. The corresponding sequences for

CYP3A64 were: Forward – 5'- CACAAACCGGAGGCCTTTT-3'; Reverse – 5'-

TCTTCCATTCTTCATCCTCAGCTA-3'; and Probe – 5'-FAM-

TCCAGTGGGATTTATGAAAAATGCCATCTCT-TAM-3'. The CYP3A4 TaqMan set was

designed to be CYP3A4 specific and was validated for specificity against plasmid DNAs

DMD#9878

containing the CYP3A4, CYP3A5, and CYP3A7 genes. The CYP3A64 set was validated against plasmid DNA containing the CYP3A64 gene. Quantitation of the target cDNA in treated samples versus DMSO (vehicle) control samples was calculated by correcting for the 18 rRNA in each sample (ΔC_t) using the equation: Fold Change = $2^{-\Delta\Delta C_t}$. 18S gene is a commonly used internal standard or housekeeping gene and we and others (Koch et al., 2002) have found it to be less sensitive to variability due to cell toxicity and induction.

Testosterone 6 β -hydroxylase and MDZ 1'-hydroxylase activities in cultured hepatocytes were measured, in triplicate, by incubating testosterone (250 μ M) or MDZ (20 μ M) with human or rhesus hepatocytes in 10 mM HEPES buffer, at 37°C, 95% humidity, and 5 % CO₂, for 15-30 min and 15-20 min, respectively. Kinetic studies with testosterone or MDZ were also conducted under similar conditions, but with various substrate concentrations. Samples from each well were transferred to a 96-well plate containing an equal volume of acetonitrile, and stored at 4°C until analysis by high pressure liquid chromatography for testosterone (Prueksaritanont et al., 2005) or liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) for MDZ (see below).

***In Vitro* Inhibition Studies.** All incubations were performed in triplicate. MDZ hydroxylation was assayed in 0.2 ml of an incubation mixture, containing 10 pmol recombinant CYP3A4 or CYP3A64, 20 mM MgCl₂, 1 mM NADPH, and 0.1 M phosphate buffer, pH 7.4. Four μ l aliquots of 50% aqueous acetonitrile (control) or various concentrations of compound A in 50% aqueous acetonitrile were co-incubated with MDZ. MDZ was prepared in 50% aqueous acetonitrile solutions from which 4 μ l aliquots were taken to generate final concentrations of 5 μ M; a value close to its K_m value (Carr et al., manuscript in review). In a separate experiment, a time-dependent inhibitory effect of compound A on CYP3A activity was examined using 75 pmol recombinant CYP3A64 or CYP3A4 preincubated with 5 μ M compound A, 1 mM NADPH,

DMD#9878

and 0.1 M phosphate buffer, pH 7.4, at 37°C for various times. An aliquot of this incubate was diluted 10-fold with buffer containing 1 mM NADPH before the addition of 20 μ M MDZ to start the reaction. To determine enzyme inactivation kinetics, similar procedures as described above were followed, but with 50 pmol CYP3A64 or CYP3A4, and various concentrations of compound A, at preincubation time of 5 min. For all experiments, incubations were conducted at 37°C and were terminated after 5 min by the addition of acetonitrile (0.2 ml per 0.2 ml incubation). The acetonitrile extracts were analyzed by an LC-MS/MS method (see below).

In Vivo Studies. All studies were reviewed and approved by the Merck Research Laboratories Institutional Animal Care and Use Committee. The *in vivo* studies were carried out in four rhesus monkeys (all male, body weight: 4-7 kg) in a crossover fashion, with at least a 2-3-week washout period. The same four animals were used in all studies described below. In the first two periods, the animals were pretreated with either vehicle (PEG400) or rifampin (18 and 1.8 mg/kg p.o., in PEG400, total volume given 5 ml), once daily for 5 days. The animals were fasted overnight before MDZ administration on day 5. On the morning of day 5, MDZ was administered intraportally (i.pv.) to a cannulated hepatic portal vein at 0.5 mg/kg/hr (using 1 ml/kg/hr) for 4 hr to monkeys and blood samples were collected via a saphenous or femoral vein at predose, 60, 120, 150, 180, 200, 220, 240 (end of i.pv. infusion), 270, 300, 360, and 420 min after MDZ administration. Plasma samples were separated immediately at 10°C and kept frozen at -20°C. Urine samples also were collected for up to 48 hr post MDZ administration. In subsequent periods, studies were conducted by i.v. administration of MDZ (0.5 mg/kg/hr for 4 hr) via a cephalic vein on day 5 in animals pretreated with the vehicle PEG400 and rifampin (18 mg/kg p.o. in PEG400) for 5 days.

Subsequently, similar studies using a lower i.pv. dose of MDZ were conducted in control monkeys and those treated with compound A, instead of rifampin. In these studies, the same

DMD#9878

animals were given a single dose of compound A at 6 mg/kg i.v. or vehicle about 10 min before the i.p.v. administration of MDZ at 0.15 mg/kg/hr for 4 hr. The lower dose of MDZ was used in order to minimize potential pharmacological activities resulting from an anticipated increase in the exposure of MDZ by compound A. For each period, blood samples were collected following MDZ administration at predose, 60, 120, 150, 180, 200, 220, 240 (end of i.p.v. infusion), 270, 300, 360, and 480 min. Plasma samples were separated immediately at 10°C and kept frozen (-20°C) prior to analysis.

Analytical Procedures for MDZ and Its Metabolites, Rifampin and Compound A.

Concentrations of MDZ, 1'-OH MDZ, 4-OH MDZ, glucuronide conjugates of the two hydroxy MDZ, rifampin and compound A in plasma and/or urine were analyzed using LC-MS/MS. Plasma samples were spiked with an internal standard (IS), diazepam, and proteins were precipitated with acetonitrile (acetonitrile:sample = 2:1 (v/v)). After centrifugation, the supernatant was subjected directly onto LC-MS/MS analysis. Urine samples were diluted with blank urine for the high concentration samples, and added with diazepam prior to analysis. The analytes were quantitated by LC-MS/MS in selective reactions monitoring (SRM) mode using an AB/MDS SCIEX API 3000 tandem mass spectrometer (Concord, Ontario, Canada) interfaced with a SCIEX Turbo IonSpray source to a Perkin Elmer Series 200 liquid chromatography system. Chromatography was accomplished on a Betasil C₁₈ column (2.1 x 50 mm, 5 µm, Keystone, Bellefonte, PA) using a mobile phase consisting of 90% acetonitrile in water (solvent B) and 10% acetonitrile in 0.02% acetic acid (pH 4.5, solvent A). The mobile phase was delivered at a flow rate of 0.5 ml/min with a linear increase of solvent B from 15% to 90% over 1 min and held at 90% for an additional minute. Equilibration was allowed for additional 1.5 min, giving a total chromatographic run time of 3.5 min. Selective reaction monitoring experiments in the positive ionization mode were performed using a dwell time of 150 ms per transition to detect

DMD#9878

the following precursor (Q1) to product (Q3) ion pairs: 326 to 291, 342 to 203, 342 to 234, 518 to 203, 518 to 234, 823 to 399, 654 to 421, and 285 to 193 for MDZ, 1'-OH MDZ, 4-OH MDZ, 1'-OH MDZ glucuronide, 4-OH MDZ glucuronide, rifampin, Compound A, and the internal standard diazepam, respectively. Calibration curves (5 - 5000 ng/ml) were prepared by plotting the appropriate peak area ratios against the concentrations of analyte in plasma using a weighted (1/x) quadratic regression. The concentration of the analyte in the unknown samples was determined by interpolation from the standard curve. For each analyte, standard curves showed satisfactory linearity and precision (<15% coefficient of variation). Due to lack of synthetic standards for glucuronide conjugates of 1'-OH MDZ and 4-OH MDZ, quantification of these glucuronides in urine samples was carried out as follows: An aliquot of a urine sample from a monkey treated with MDZ was hydrolyzed by β -glucuronidase (5000 unit/ml urine, 37°C for 24 hr). Analysis by LC-MS confirmed the complete hydrolysis of the conjugates to 1'-OH and 4-OH MDZ, which were then quantified with their respective standard curves. The difference in the two hydroxy MDZ amounts determined after and prior to hydrolysis was considered as the amounts of the respective glucuronides in that urine sample. This quantified urine sample was kept at -20°C for the generation of standard curves for those conjugates in all urine samples.

Data Analysis. Apparent K_m and V_{max} values were estimated using a nonlinear regression program (Enfit from Biosoft, Ferguson, MO). The CL_{int} values were estimated by dividing V_{max} by K_m . The concentration of rifampin producing a 50% increase in MDZ 1'-hydroxylase activity (EC_{50}) was determined using non-linear regression analysis (PCNONLIN, Scientific Consulting, Cary, NC), based on the following relationship:

$$E = E_{max} \times C / (C + EC_{50})$$

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

DMD#9878

The kinetic parameters (K_{inact} and K_I) of CYP3A4/CYP3A64 inactivation were determined using standard procedures as described previously (Prueksaritanont et al., 1998; Chiba et al., 1995).

The area under the plasma concentration-time profile (AUC_{0-last}) was calculated from time zero to the last detectable sampling time using the linear trapezoidal rule. The apparent terminal half-life ($t_{1/2}$) was estimated by dividing 0.693 by the elimination rate constant determined using least-squares regression analysis of the log-linear portion of the MDZ plasma concentration-time data. Clearance (CL) values for MDZ were calculated as the i.v. dose divided by the AUC from time zero to infinity (AUC_{0-inf}). Hepatic availability (F_h) was estimated by dividing AUC of MDZ obtained following i.p.v. administration to that obtained by i.v. administration. Volume of distribution at steady-state (V_{dss}) values were estimated by conventional moment analysis as i.v. dose multiplied by the first moment of the plasma concentration-time profile (AUMC) and divided by (AUC_{0-inf})². The peak plasma concentration (C_{max}) was determined by observation.

Statistical Analysis. Statistical analysis was performed using a two tailed pair *t* test. A *p* value of <0.05 was considered statistically significant.

Results

***In Vitro* Induction of CYP3A64 and CYP3A4 by Rifampin.** Cultured hepatocytes, currently the accepted and recommended method for studying CYP induction (LeCluyse, 2001; Bjornsson et al., 2003) were used in this study. Under the present experimental conditions, rifampin, at 10 μ M, induced markedly (~5-10-fold) the mRNA level of CYP3A64, and modestly (~1.5 – 2 fold) MDZ 1'-hydroxylase activity, in all six monkey hepatocyte preparations (Fig. 1A). The relatively low fold induction at the functional activity level is consistent with a previous observation in this species (Wen et al., 2002). Under similar conditions, rifampin, at 10 μ M, also appreciably increased CYP3A4 mRNA expression (~6-14-fold, Fig. 1B) and activity (~3-10-fold), as measured by testosterone 6 β -hydroxylation (Fig. 1B) in all six human hepatocyte preparations. In some subjects, the MDZ 1'-hydroxylase activity also was measured and similar results to those of testosterone 6 β -hydroxylase were obtained (not shown). These results were consistent with those reported earlier with rifampin in cultured human hepatocytes (LeCluyse, 2001; Luo et al., 2003).

Further studies on effects of various concentrations of rifampin revealed that rifampin was a potent inducer of CYP3A64. In hepatocyte preparations from two individual monkeys, the mean (range) EC₅₀ for CYP3A64 mRNA, and MDZ 1'-hydroxylase activity were about 0.5 (0.4 – 0.6) and 0.2 (0.2 – 0.2) μ M, respectively. The corresponding mean (range) E_{max} values were 6- (6 – 6) and 2.2- (1.8 – 2.7) fold, respectively, over control values (Fig. 2A). Similar results also were obtained with the testosterone 6 β -hydroxylase activity (Fig. 2A). Additional detailed studies on kinetics of MDZ metabolism in monkey hepatocytes suggested that 10 μ M rifampin treatment had little effect on the apparent K_m, but increased (~2-fold) the V_{max} for both 1'- and 4-OH MDZ (Fig. 2B). Values for the K_m for 1'-OH MDZ and 4-OH MDZ formation ranged from 1.5 – 2 μ M and 3.3 – 4.8 μ M, respectively, in both the vehicle treated and rifampin treated monkey hepatocytes. The corresponding V_{max} values were 0.011 and 0.026 nmol/min/well for 1'-OH

DMD#9878

MDZ and 0.003 and 0.011 nmol/min/well for 4-OH MDZ, for the vehicle control and rifampin treatment, respectively. Consequently, the resulting intrinsic clearance (V_{\max}/K_m) was increased by about 2-fold, comparable to the aforementioned E_{\max} value obtained with a single concentration (20 μ M) of MDZ (Fig. 1A).

***In Vitro* Inhibition of CYP3A64 and CYP3A4 by Compound A.** In incubations with recombinant CYP3A64 and CYP3A4, compound A suppressed the formation of 1'-OH MDZ in a concentration dependent fashion, with comparable potency; the IC_{50} values (~ 1 μ M) determined using 5 μ M MDZ substrate concentration were essentially identical between the 2 systems (data not shown). The inhibitory effect of compound A on CYP3A64-mediated MDZ 1'-hydroxylase activity was also time-dependent, similar to that obtained with CYP3A4 (Figs. 3A and B) and consistent with the earlier finding in human liver microsomes (Chiba et al., 1995). Enzyme kinetics studies suggested that compound A was a potent and efficient mechanism-based inhibitor of both CYP3A64 and CYP3A4, as measured by MDZ 1'-hydroxylase activity, relative to known mechanism-based inhibitors (Ernest et al., 2005); values for K_I were 0.25 and 0.4 μ M and k_{inact} were 0.4 and 0.7 min^{-1} , for CYP3A64 and CYP3A4, respectively. Interestingly, comparable results also were obtained in our preliminary study with monkey and human liver microsomes; values for K_I were 0.15 and 0.26 μ M, and k_{inact} were 0.4 and 0.7 min^{-1} , respectively (data not shown), consistent with the notion that compound A is a selective CYP3A inhibitor (Chiba et al., 1995).

Effects of Rifampin on the Pharmacokinetics of MDZ After Intravenous Administration to Monkeys. Following intravenous infusion of MDZ (0.5 mg/kg/hr for 4 hr) to control animals, plasma concentrations of MDZ appeared to reach a steady-state level of about 0.8 to 1.0 μ M at about 2 hr post dose, and declined relatively quickly after the infusion stopped (Fig. 4A), with $t_{1/2}$ of about 1 hr (Table 1). In these monkeys, plasma clearance values ranged

DMD#9878

from 24 to 32 ml/min/kg, with a mean value of 27 ml/min/kg (Table 1). The major metabolite 1'-OH MDZ was detectable in plasma plasma, although with relatively low levels, in all four monkeys (Fig. 4B). The AUC in all four monkeys (Fig. 4B); the AUC values of 1'-OH MDZ ranged from 0.2 to 0.9 $\mu\text{M}\cdot\text{hr}$, representing about 13% (range: 5 to 22%) of those for the parent MDZ (Table 1). Plasma levels of 4-OH MDZ were near detectable limit and thus not shown, in control monkeys. Analysis of urine samples revealed that the total urinary recovery of MDZ and its metabolites were about 67% of the i.v. dose, with 1'-OH MDZ glucuronide representing the major metabolite (65% of the dose) and 4-OH MDZ being the minor pathway (<1% of the dose) (Table 1). Noteworthy that complete assessment of the *in vivo* MDZ metabolism in rhesus monkeys was not possible since recoveries of MDZ and its metabolites in feces via biliary excretion were not determined in this study. Nevertheless, the urinary analysis suggested that in rhesus monkeys, MDZ underwent extensive metabolism via oxidation (at least 67%) followed by glucuronidation, and that urinary excretion was the major (~70%) elimination pathway.

Treatment with rifampin for 5 days at 18 mg/kg p.o. did not significantly affect the i.v. plasma profiles (Fig. 4A) or pharmacokinetic parameters of MDZ; values for AUC, V_{dss} , CL and $t_{1/2}$ of MDZ were comparable between the control and rifampin treated animals (Table 1). However, rifampin significantly decreased plasma levels of 1'-OH MDZ (Fig. 4B); values for 1'-OH MDZ were decreased to approximately 1% of that of MDZ, representing about 10% of the control value (Table 1). A metabolite switching phenomenon was not apparent as a similar decreasing trend in plasma levels of 4-OH MDZ also was observed in rifampin treated vs control animals (data not shown).

Effects of Rifampin on the Pharmacokinetics of MDZ after Intraportal Vein

Administration to Monkeys. Shown in Figs. 5A and 5B are mean plasma profiles of MDZ and its metabolite 1'-OH MDZ, respectively, following i.p.v. administration of MDZ (0.5 mg/kg/hr for 4 hr) to rhesus monkeys pretreated with vehicle or rifampin (18 and 1.8 mg/kg p.o.) for 5 days.

DMD#9878

In control animals, MDZ plasma levels obtained after i.p.v. administration were significantly lower than those observed following i.v. administration, with an apparent steady-state concentration of about 0.3 μM (range 0.1 to 0.4 μM) and AUC of 0.7 $\mu\text{M}\cdot\text{hr}$ (Fig. 5A, Table 2). As a consequence, the hepatic availability, F_h , of MDZ was low (16%, Table 2), suggesting extensive hepatic first pass elimination of MDZ in control monkeys. Consistent with this, ratios between plasma levels of 1'-OH MDZ and MDZ following i.p.v. administration of MDZ (Fig. 5B) were higher than those obtained following the i.v. dosing; a mean value for 1'-OH/MDZ AUC ratio was about 2 (Table 2).

Unlike the observation with the i.v. administration, treatment of rifampin at 18 mg/kg p.o. for 5 days caused a pronounced decrease (~90%) in MDZ AUC after i.p.v. administration of MDZ (Table 2). Consequently, the F_h of MDZ was markedly decreased to 1% by rifampin treatment. A similar magnitude of interactions also was obtained following administration of rifampin at 1.8 mg/kg p.o. for 5 days (Fig. 5A; Table 2), suggesting that an apparent maximal induction effect of rifampin was reached at the doses employed in this study. As was the case with the i.v. dosing, marked decreases in 1'-OH MDZ AUC also were observed in monkeys treated with rifampin, either at 18 or 1.8 mg/kg (Fig. 5B, Table 2). However, the AUC ratios between 1'-OH MDZ and MDZ in these animals, at both doses, although slightly higher than (~20-43%), were not statistically significant from control values (Table 2).

In monkeys pretreated with rifampin, plasma concentrations of rifampin were maintained above 5 and 0.2 μM , following 18 and 1.8 mg/kg doses, respectively, over a period of 6 hr after the last dose (5th dosing day) (Fig. 6). As shown in Table 2, systemic exposures of rifampin were increased as doses increased, although more than dose proportional; plasma levels of rifampin reached C_{max} of about 15 μM and 0.3 μM , and values for $\text{AUC}_{0-7\text{hr}}$ of rifampin were 67 and 1.2 $\mu\text{M}\cdot\text{hr}$, after 18 and 1.8 mg/kg, respectively (Table 2). Plasma concentrations of rifampin

DMD#9878

obtained at the 18 mg/kg dose were comparable to those reported following a therapeutic dose in humans (Sanofi-Aventis, 2003).

Effects of Compound A on the Pharmacokinetics of MDZ after Intraportal Vein

Administration to Rhesus Monkeys. Following MDZ administration (0.15 mg/kg/hr) to control animals, plasma levels of MDZ were relatively low (Fig. 7), with mean AUC value of 0.03 $\mu\text{M}\cdot\text{hr}$ (Table 3). Consistent with the finding with the 0.5 mg/kg/hr MDZ dose, 1'-OH MDZ plasma levels were substantial; the AUC ratio between 1'-OH MDZ and MDZ was about 1.6 (Fig. 7, Table 3).

Treatment with compound A caused marked increases in the AUC values of both MDZ and 1'-OH MDZ by 10-fold and 4-fold, respectively (Fig. 7, Table 3), resulting in statistically significant decreases in the 1'-OH MDZ/MDZ AUC ratio to about 60% (Table 3). Also, a similar increasing trend in plasma levels of 4-OH MDZ were observed in animals treated with compound A (data not shown). At the dose of compound A used in this study, plasma levels of compound A were above 0.5 μM for about 6 hr (Fig. 5), with a mean $\text{AUC}_{0-8\text{hr}}$ value of 7 $\mu\text{M}\cdot\text{hr}$ (Table 3).

Discussion

In the present *in vitro* studies, rifampin was characterized as an equipotent inducer of both rhesus monkey CYP3A64 and human CYP3A4, while compound A was equally efficient as an inactivator of CYP3A64 and CYP3A4. Subsequent pharmacokinetic interaction studies with MDZ, showed that *in vivo*, rhesus monkey CYP3A64 also was susceptible to induction by rifampin and inhibition by compound A. As is the case for CYP3A4 in humans, the *in vivo* clearance mechanism of MDZ in rhesus monkeys likely is primarily via CYP3A64, qualifying MDZ pharmacokinetics as an *in vivo* CYP3A64 probe. This conclusion is supported by 1) the finding that MDZ underwent extensive metabolism (at least 67%) in rhesus monkeys, with 1'-OH MDZ constituting the major metabolic pathway (Table 1), similar to *in vivo* observations in humans (Heizman and Ziegler, 1981), and 2) the report by Carr et al. (manuscript in review) demonstrating MDZ as a good *in vitro* probe substrate for CYP3A64, similar to CYP3A4. Additionally, our immunoinhibition studies using highly selective anti-human CYP3A4 monoclonal antibody (Mei et al., 1999; Carr et al., manuscript in review) showed a near complete inhibition (>90%) of the major 1'-OH MDZ and minor 4-OH MDZ metabolites, in both monkey and human liver microsomes (data not shown). Nevertheless, possible contributions of other rhesus CYP3A enzymes to MDZ metabolism could not be completely ruled out. Pharmacokinetically, MDZ was characterized as a high clearance compound in rhesus monkeys, based on a blood/plasma partition ratio for MDZ of 0.6 (data not shown) and a mean CL of 27 ml/min/kg (Table 1), yielding a mean blood clearance (about 45 ml/min/kg) approaching the hepatic blood flow reported for this species (50-55 ml/min/kg). Interestingly, this *in vivo* clearance observation also is consistent with the high basal MDZ 1'-hydroxylase activity observed *in vitro* with CYP3A64.

DMD#9878

The MDZ and rifampin interaction results provided some useful insights into the *in vitro* – *in vivo* CYP induction relationship. The finding that rifampin did not significantly affect the i.v. kinetics of MDZ (CL or $t_{1/2}$), while markedly decreased F_h (~16-fold), is in line with the concept (Wilkinson, 1987) that the i.v. pharmacokinetics of high clearance compounds are much less sensitive to changes in their intrinsic hepatic clearances, while their F_h (and oral pharmacokinetics) are expected to be significantly affected even with a small change in the intrinsic clearance (V_{max}/K_m of ~2-fold based on *in vitro* studies in this case). Indeed, a comparably large magnitude decrease in F_h (~10-fold) was estimated using the well known parallel-tube model ($F_h = 1 - (CL_h/Q_h)$; $CL_h = Q_h [1 - e^{(-f_u CL_{int}/Q_h)}]$), based on the 2-fold change in CL_{int} , and assuming the hepatic blood clearance (CL_h) of MDZ and hepatic blood flow (Q_h) of 45 and 50 ml/min/kg, respectively, and an unbound fraction of MDZ in monkey plasma (f_u) of 0.2 (data not shown). The parallel-tube model, which has provided reasonable estimates for MDZ and other drugs with high extraction ratio (Higashikawa et al., 1998), also predicted a disproportionate change in F_h (~30-fold) with a mere 2.5-fold change in CL_{int} . Thus, this finding further supports the view that the likely extent of *in vivo* interaction could not be readily gauged based solely on *in vitro* V_{max}/K_m or E_{max} values, but also the *in vivo* pharmacokinetic characteristics of the probe. Additionally, the present results suggest that the *in vitro* EC_{50} value (the effective concentration for 50% maximal induction) is a helpful parameter in determining a range of plasma concentrations of inducers that would likely cause significant *in vivo* drug interactions. This conclusion is based on the finding that the apparent maximal induction effect of rifampin was observed in association with rifampin's C_{max} of ~0.3 μ M, which was slightly higher than the EC_{50} value of 0.2 μ M obtained in monkey hepatocytes. Given the fact that rifampin is a substrate of a human hepatic transporter, OATP (Tirona et al., 2003), a possibility exists that after the 1.8 mg/kg dose, the unbound intracellular concentrations of rifampin in monkey liver would exceed those observed in plasma (0.06 μ M unbound concentration, based on f_u of 20%), and consequently reached the *in vitro* EC_{50} value.

DMD#9878

Similarly, the *in vivo* relevance of the *in vitro* inhibition of CYP3A64 was demonstrated in rhesus monkeys. The finding that compound A markedly increased the systemic exposure of the i.p.v. administered MDZ is consistent with inhibition of CYP3A64-mediated hepatic metabolism of MDZ in this species. Notably, this inhibition effect was observed at plasma concentrations of compound A over the 6-hr period of about 0.5 to 1 μM , which corresponded to unbound plasma concentrations of approximately 0.1 to 0.2 μM (based on 20% unbound fraction obtained using monkey plasma - data not shown) and exceeded its K_I value obtained *in vitro* with CYP3A64. Although the single dosing regimen used in this study may not be sufficient to produce the maximal level of enzyme inactivation by compound A, the magnitude increase in the i.p.v. AUC of MDZ by compound A was nevertheless close to an anticipated range based on the *in vitro* k_{inact} and K_I obtained in this study and using a quantitative prediction method proposed for mechanism-based inactivators by Ernest et al. (2005).

In this study, the pharmacokinetics of 1'-OH MDZ also was monitored, but unfortunately limited information was attained. Unlike the *in vitro* metabolite formation kinetics, *in vivo* metabolite kinetics is known to be quite complex, dependent not only on its formation rate, but also its elimination rate as well as the exposure of its parent (Houston, 1982). The finding that the exposure to 1'-OH MDZ was markedly decreased with a comparable decrease in 1'-OH MDZ/MDZ AUC ratios after i.v. administration of MDZ to monkeys pretreated with rifampin suggested that rifampin might also induce a major elimination process of 1'-OH MDZ, presumably glucuronidation. This suggestion is in agreement with published reports that rifampin is an inducer for UGT (Li et al., 1999; Soars et al., 2004). However, the reasons for the different observation obtained following the i.p.v. administration of MDZ (decreased MDZ and 1'-OH MDZ exposure, but not the 1'-OH MDZ/MDZ ratio) are unclear. Whether this apparent discrepancy is partly related to potential induction of the glucuronidation of 1'-OH MDZ during

DMD#9878

hepatic first pass by rifampin remains to be investigated. Regarding the *in vivo* effect of compound A, the finding of significantly decreased 1'-OH MDZ/MDZ AUC ratio, although accompanied by increased 1'-OH MDZ exposure, is explainable by the more markedly increase in MDZ exposure, and consistent with the conclusion that compound A inhibited primarily the hepatic formation of 1'-OH MDZ via CYP3A64 inhibition.

Analogous to rhesus monkeys, an apparent *in vitro-in vivo* agreement also was obtained with MDZ and rifampin in humans. In clinical studies, rifampin has been reported to significantly increase the i.v. CL (2-fold) and decrease oral exposure of MDZ (20-40-fold) (Backman et al., 1996, 1998; Kharasch et al., 2004). The effect on CL is consistent with the *in vitro* induction data observed in human hepatocytes, as well as the fact that MDZ exhibits a moderate CL in humans (unlike monkeys), while that on the oral AUC could be attributable additionally to changes in the hepatic first pass as well as the induction of CYP3A4-mediated intestinal first pass of MDZ (Thummel et al., 1994; Tsunoda et al., 1999). In this regard, the i.p.v. administration was used in the present monkey study to avoid potential complications from incomplete absorption and intestinal enzyme induction or inhibition. This allowed for a direct comparison with the present *in vitro* studies using liver preparations. Although not studied, the inhibitory effect of compound A on the MDZ pharmacokinetics in humans is also expected to be comparable to that observed in the present rhesus monkey study, based on almost identical *in vitro* CYP3A64 and CYP3A4 data, and the clinical observations that a similar magnitude of interactions have been reported for MDZ when co-administered with mechanism-based inhibitors with comparable k_{inact} and K_I values (Ernest et al., 2005).

In addition to rifampin and compound A presented in this investigation, our in-house studies with a limited number of compounds (Merck Research Labs, data on file) also showed species similarities in induction and inhibition profiles between rhesus monkey CYP3A64 and

DMD#9878

human CYP3A4. In the case of rifampin, the enzyme inducing effect in rhesus monkeys appeared to be mediated via the pregnane X receptor, PXR, (Merck Research Labs, data on file), similar to humans (Gibson et al., 2002; Luo et al., 2002). Nevertheless, species differences in CYP responses between rhesus monkeys and humans remain a possibility for other compounds, considering the well documented marked species differences, especially between rodents and humans in response to CYP3A inducers (Gibson et al., 2002).

In conclusion, the present investigation showed that *in vitro*, rhesus monkey CYP3A64 was susceptible to induction by rifampin and inhibition by compound A, resembling observations with human CYP3A4. With the use of MDZ as an *in vivo* probe for CYP3A64, the pharmacokinetic consequences of the *in vitro* findings and their corresponding *in vitro* – *in vivo* relationships were demonstrated in this species. With respect to CYP induction, both the *in vitro* E_{max} and EC_{50} values were valuable, when considered in combination with the ADME or pharmacokinetics characteristics of the probe and the inducer as well as route of administration of the probe, for assessing the likely extent of *in vivo* interaction. Finally, our results suggest that these *in vitro* and *in vivo* rhesus monkey models, when used in conjunction with *in vitro* human systems, may serve as valuable preclinical tools to help provide a basis for extrapolating *in vitro* human data to clinical outcomes and a mechanistic insight for the interpretation of drug interactions observed during early/late drug development processes.

DMD#9878

Acknowledgments

We thank Ms. J. Brunner, K. Michel and J. Adelsburger for animal experiments.

DMD#9878

References

- Backman JT, Olkkola T and Neuvonen PJ (1996) Rifampin drastically reduces plasma concentrations and effects of oral midazolam. *Clin Pharmacol Ther* **59**:7-13.
- Backman JT, Kivistö KT, Olkkola T and Neuvonen PJ (1998) The area under the plasma concentration-time curve for oral midazolam is 400-fold larger during treatment with itraconazole than with rifampin. *Eur J Clin Pharmacol* **54**:53-58.
- Bjornsson TD, Callaghan JT, Einolf HJ, Fisher V, Gan L, Grimm S, Kao J, King SP, Miwa G, Ni L, Kumar G, McLeod J, Obach SR, Roberts S, Roe A, Shah A, Snikeris F, Sullivan JT, Tweedie D, Vega JM, Walsh J and Wrighton SA (2003) The conduct of *in vitro* and *in vivo* drug-drug interaction studies: A PhRMA perspective. *J Clin Pharmacol* **43**:443-469.
- Chiba M, Nishimi JA and Lin JH (1995) Potent and selective inactivation of human liver microsomal cytochrome P-450 isoforms by L-754,394, an investigational human immune deficiency virus protease inhibitor. *J Pharmacol Exp Ther* **275**:1527-1534.
- Dich J and Grunnet N (1990) Primary cultures of rat hepatocytes. In: JW Pollard, Editor, *Methods in Molecular Biology* 5, The Humana Press Inc., Clifton, pp. 161–176.
- Dresser GK, Spence JD and Bailey DG (2000) Pharmacokinetic-pharmacodynamic consequences and clinical relevance of cytochrome P450 3A4 inhibition. *Clin Pharmacokinet* **38**:41-57.
- Ernest CS, Hall SD and Jones DR (2005) Mechanism-based inactivation of CYP3A by HIV protease inhibitors. *J Pharmacol Exp Ther* **312**:583-591.
- Gibson GG, Plant NJ, Swales KE, Ayrton A and El-Sankary W (2002) Receptor-dependent transcriptional activation of cytochrome P450 3A genes: induction, mechanisms, species differences and interindividual variations in man. *Xenobiotica* **32**:165-206.
- Heizmann P and Ziegler WH (1981) Excretion and metabolism of ¹⁴C-midazolam in humans following oral dosing. *Arzneim-Forsch* **31**:2220-2223.

DMD#9878

- Higashikawa F, Murakami T, Kaneda T, and Takano M (1999) In-vivo and in-vitro metabolic clearance of midazolam, a cytochrome P450 3A substrate, by the liver under normal and increased enzyme activity in rats. *J Pharm Pharmacol* **51**:405-410.
- Houston JB (1982) Drug metabolite kinetics. *Pharmac Ther* **15**:521-552.
- Houston JB and Galetin A (2003) Progress towards prediction of human pharmacokinetic parameters from *in vitro* technologies. *Drug Metab Rev* **35**:393-415.
- Igarashi T, Sakuma T, Isogal M, Nagata R and Kamataki T (1997) Marmoset liver cytochrome P450s: study for expression and molecular cloning of their cDNAs. *Arch Biochem Biophys* **339**:85-91.
- Ito K, Ogihara K, Kanamitsu S-I, and Itoh T (2003) Prediction of the *in vivo* interaction between midazolam and macrolides based on *in vitro* studies using human liver microsomes. *Drug Metab Dispos* **31**:945-954.
- Ito K, Brown HS and Houston JB (2004) Database analyses for the prediction of *in vivo* drug-drug interactions from *in vitro* data. *Br J Clin Pharmacol* **57**:473-486.
- Kanazu T, Yamaguchi Y, Okamura N, Baba T and Koike M (2004) Model for the drug-drug interaction responsible for CYP3A enzyme inhibition. I: evaluation of cynomolgus monkeys as surrogates for humans. *Xenobiotica* **34**:391-402.
- Kharasch ED, Hoffer C, Whittington D and Sheffels P (2004) Role of hepatic and intestinal cytochrome P450 3A and 2B6 in the metabolism, disposition, and miotic effects of methadone. *Clin Pharmacol Ther* **76**:250-269.
- Koch I, Weil R, Wolbold R, Brockmüller J, Hustert E, Burk O, Nuessler AC, Neuhaus P, Eichelbaum M, Zanger UM, and Wojnowski L (2002) Interindividual variability and tissue-specificity in the expression of cytochrome P450 3A mRNA. *Drug Metab Dispos* **30**:1108–1114.
- LeCluyse EL (2001) Human hepatocyte culture systems for the *in vitro* evaluation of cytochrome P450 expression and regulation. *Eur J Pharm Sci* **13**:343-368.

DMD#9878

- Lesar TS, Briceland L and Stein DS (1997) Factors related to errors in medication prescribing. *J Am Med Ass* **277**:312-317.
- Li AP, Hartman NR, Lu C, Collins JM and Strong JM (1999) Effects of cytochrome P450 inducers on 17 α -ethinyloestradiol (EE2) conjugation by primary human hepatocytes. *Br J Clin Pharmacol* **48**:733-742.
- Lin JH, Chiba M, Chen I-W, Vastag KJ, Nishimi JA, Dorsey BD, Michelson SR and McDaniel SL (1995) Time- and dose-dependent pharmacokinetics of L-754,394, an HIV Protease inhibitor, in rats, dogs and monkeys. *J Pharmacol Exp Ther* **274**:264-269.
- Luo G, Cunningham M, and Kim S., Burn T, Lin J, Sinz M, Hamilton G, Rizzo C, Jolley S, Gilbert D, Downey A, Mudra D, Graham R, Carroll K, Xie J, Madan A, Parkinson A, Christ D, Selling B, LeCluyse E and Gan L-S (2002) CYP3A4 induction by drugs: correlation between a pregnane X receptor reporter gene assay and CYP3A4 expression in human hepatocytes. *Drug Metab Dispos* **30**:795-804.
- Mei Q, Tang C, Assang C, Lin Y, Slaughter D, Rodrigues AD, Baillie TA, Rushmore TH and Shou M (1999) Role of a potent inhibitory monoclonal antibody to cytochrome P-450 3A4 in assessment of human drug metabolism. *J Pharmacol Exp Ther* **291**:749-759.
- Moldeús P, Hogberg J and Orrenius S (1978) The isolation and use of liver cells. *Methods Enzymol.* **52**:60-71.
- Niemi M, Backman JT, Fromm MF, Neuvonen J and Kivistö KT (2003) Pharmacokinetic interactions with rifampin: clinical relevance. *Clin Pharmacokinet* **42**:819-850.
- Prueksaritanont T, Zhao J, Ma B, Roadcap B, Tang C, Qiu Y, Liu L, Lin JH, Pearson PG and Baillie TA (2002) Mechanistic studies on the metabolic interactions between gemfibrozil and statins. *J Pharmacol Exp Ther* **301**:1042-1051.
- Prueksaritanont T, Richards KM, Qiu Y, Strong-Basalyga K, Miller A, Li C, Eisenhandler R and Carlini EJ (2005) Comparative effects of fibrates on drug metabolizing enzymes in human hepatocytes. *Pharm Res* **22**:71-78.

DMD#9878

Sanofi-Aventis (2003) Prescribing information on Rafadin (Rifampin). Physicians' Desk

Reference, Medical Economics Co. pp 759-762, Montvale, NJ.

Sharer JE, Shipley LA, Vandenbranden MR, Binkley SN and Wrighton SA (1995) Comparisons of phase I and phase II *in vitro* hepatic enzyme activities of human, dog, rhesus monkey, and cynomolgus monkey. *Drug Metab Dispos* **23**:1231-1241.

Soars MG, Petullo DM, Eckstein JA, Kasper SC and Wrighton SA (2004) An assessment of UDP-glucuronosyltransferase induction using primary human hepatocytes. *Drug Metab Dispos* **32**:140-148.

Thummel KE, Shen DD, Podoli TD, Kunze KL, Trager WF, Bacchi CE, Marsh CL, McVicar JP, Barr DM, Perkins JD and Carithers RL (1994) Use of midazolam as a human cytochrome P450 3A probe II. Characterization of inter- and intraindividual hepatic CYP3A variability after liver transplantation. *J Pharmacol Exp Ther* **271**:557-566.

Thummel KE and Wilkinson GR (1998) In vitro and *in vivo* drug interactions involving human CYP3A. *Annu Rev Pharmacol Toxicol* **38**:389-430.

Tirona RG, Leake BF, Wolkoff AW and Kim RB (2003) Human organic anion transporting polypeptide-C (SLC21A6) is a major determinant of rifampin-mediated pregnane X receptor activation. *J Pharmacol Exp Ther* **304**:223-228.

Tsunoda SM, Velez RL, von Moltke LL and Greenblatt DJ (1999) Differentiation of intestinal and hepatic cytochrome P450 3A activity with use of midazolam as an *in vivo* probe: effect of ketoconazole. *Clin Pharmacol Ther* **66**:461-471.

Tucker GT, Houston JB and Huang S-M (2001) Optimizing drug development: strategies to assess drug metabolism/transporter interaction potential-towards a consensus. *Br J Clin Pharmacol* **52**:107-117.

Weaver RJ (2001) Assessment of drug-drug interactions: concepts and approaches. *Xenobiotica* **31**:499-538.

DMD#9878

- Wen YH, Sahi J, Urda E, Kulkarni S, Rose K, Zheng X, Sinclair JF, Cai H, Storm SC and Kostrubsky V (2002) Effects of bergamotin on human and monkey drug-metabolizing enzymes in primary cultured hepatocytes. *Drug Metab Dispos* **30**:977-984.
- Wilkinson GR (1987) Clearance approaches in pharmacology. *Pharmacol Rev* **39**:1-47.
- Worboys PD and Carlile DJ (2001) Implications and consequences of enzyme induction on preclinical and clinical drug development. *Xenobiotica* **31**:539-556.

DMD#9878

Footnotes

This work was conducted at Merck Research Laboratories, West Point, PA

Reprint requests should be sent to:

Thomayant Prueksaritanont, Ph.D.

Department of Drug Metabolism

WP75-100

Merck Research Laboratories

West Point, PA 19486

DMD#9878

Figure Legends

FIG. 1. Effect of 10 μ M rifampin on CYP3A64 or CYP3A4 mRNA expression and testosterone 6 β -hydroxylase and/or MDZ 1'-hydroxylase activity in rhesus monkey (A) and human (B) hepatocytes from 6 different individuals.

Results are mean of triplicate determinations. Testosterone 6 β -hydroxylase or MDZ 1'-hydroxylase activity was determined using 250 μ M testosterone or 20 μ M MDZ, respectively. N/A = data not available.

FIG. 2. Effect of various rifampin concentrations on CYP3A64 mRNA expression, and testosterone 6 β -hydroxylase and MDZ 1'-hydroxylase activity in rhesus monkey hepatocytes isolated from 2 individual subjects (A), and kinetics of 1'-OH and 4-OH MDZ formation in rhesus monkey hepatocytes treated with vehicle (DMSO, control) or rifampin (10 μ M) for 48 hr (B).

Results shown in Fig. 2A are mean of two individual subjects, each determined in triplicate, using 250 μ M testosterone or 20 μ M MDZ as a marker substrate. Results shown in Fig. 2 B are mean \pm SD of triplicate determinations from one individual subject. Formation of 1'-OH and 4-OH MDZ was determined following incubation with various concentrations of MDZ for 20 min.

FIG. 3. Concentration- and time-dependent inhibitory effect of compound A on MDZ 1'-hydroxylase activity mediated with recombinant CYP3A64 (A) and CYP3A4 (B).

Results are expressed as percentage of control values (means of triplicate determinations), and were obtained following preincubation of compound A, NADPH and liver microsomes for at 37°C, and at various preincubation times, the incubation mixture was diluted 10-fold with buffer before the addition of MDZ (20 μ M) and NADPH to start the reaction.

DMD#9878

FIG. 4. Mean plasma concentration-time profiles of MDZ (A) and 1'-OH MDZ (B) following i.v. administration of MDZ (0.5 mg/kg/hr for 4 hr) to rhesus monkeys pretreated with vehicle or rifampin (18 mg/kg p.o. once daily) for 5 days. Results are mean \pm SD, n=4.

FIG. 5. Mean plasma concentration-time profiles of MDZ (A) and 1'-OH MDZ (B) following i.p.v. administration of MDZ (0.5 mg/kg/hr for 4 hr) to rhesus monkeys pretreated with vehicle or rifampin (18 or 1.8 mg/kg p.o. once daily) for 5 days. Results are mean \pm SD, n=4.

FIG. 6. Mean plasma concentration-time profiles of rifampin following oral administration of rifampin at 18 mg/kg or 1.8 mg/kg for 5 days, and of compound A following i.v. administration of compound A at 6 mg/kg single dose. Results are mean \pm SD, n=4.

FIG. 7. Mean plasma concentration-time profiles of MDZ and 1'-OH MDZ following i.p.v. administration of MDZ (0.15 mg/kg/hr for 4 hr) to rhesus monkeys pretreated with vehicle or compound A (6 mg/kg i.v. single dose). Results are mean \pm SD, n=4.

DMD#9878

TABLE 1

Pharmacokinetic parameters of MDZ and its metabolites following intravenous administration of MDZ (0.5 mg/kg/hr for 4 hr) to rhesus monkeys pretreated with vehicle (PEG400) or rifampin (18 mg/kg p.o.) for 5 days.

Compound measured	PK Parameters	Vehicle Control	Rifampin 18 mg/kg
			<u>% of control</u>
<u>Plasma</u>			
MDZ	AUC _{0-last} , μ M.hr	3.79 \pm 0.39	4.10 \pm 0.78 109 \pm 13 (97 - 123)
	CL, ml/min/kg	26.6 \pm 3.4	25.2 \pm 5.6 94 \pm 13 (80 - 104)
	V _{dss} , L/kg	1.77 \pm 0.50	1.70 \pm 0.69 93 \pm 18 (67 - 107)
	t _{1/2} , hr	0.9 \pm 0.2	0.8 \pm 0.3 90 \pm 28 (65 - 130)
1'-OH MDZ	AUC _{0-last} , μ M.hr	0.52 \pm 0.32	0.055 \pm 0.025* 13 \pm 7 (6 - 23)
1'-OH MDZ/MDZ Ratio	AUC _{0-last}	0.13 \pm 0.07	0.013 \pm 0.004* 12 \pm 6 (5 - 18)
<u>Urine</u>			
MDZ	Recovery, % of dose	0.03 \pm 0.01	
1'-OH MDZ	Recovery, % of dose	1.6 \pm 1.3	
1'-OH MDZ glucuronide	Recovery, % of dose	64.8 \pm 9.0	
4-OH MDZ (unconjugate + conjugate)	Recovery, % of dose	0.3 \pm 0.1	

Values are Mean \pm SD, n=4.

Values in parentheses are range.

*Indicates statistically significant difference from control ($p < 0.05$).

TABLE 2

Pharmacokinetic parameters of MDZ and 1'-OH MDZ following intra-hepatic portal vein administration of MDZ (0.5 mg/kg/hr for 4 hr) to rhesus monkeys pretreated with vehicle (PEG400) or rifampin (1.8 or 18 mg/kg p.o.) for 5 days.

Compound measured	PK Parameters	Vehicle Control	Rifampin 18 mg/kg	Rifampin 1.8 mg/kg
			<i>% of control</i>	<i>% of control</i>
MDZ	AUC _{0-last} , $\mu\text{M}\cdot\text{hr}$	0.66 \pm 0.60	0.037 \pm 0.03* 10 \pm 4 (6 - 16)	0.059 \pm 0.04* 11 \pm 3 (7 - 15)
	AUC _{0-inf} , $\mu\text{M}\cdot\text{hr}$	0.70 \pm 0.66	0.045 \pm 0.04* 9 \pm 7 (4 - 17)	0.072 \pm 0.05* 12 \pm 3 (8 - 16)
	F _h	0.16 \pm 0.13	0.010 \pm 0.01* 8 \pm 7 (2 - 19)	N/A**
	t _{1/2} , hr	0.8 \pm 0.2	1.0 \pm 0.5 132 \pm 59 (74 - 213)	
1'-OH MDZ	AUC _{0-inf} , $\mu\text{M}\cdot\text{hr}$	0.923 \pm 0.38	0.071 \pm 0.06* 9 \pm 7 (3 - 15)	0.133 \pm 0.08* 15 \pm 6 (7 - 20)
1'-OH MDZ/MDZ Ratio	AUC _{0-inf} , $\mu\text{M}\cdot\text{hr}$	2.0 \pm 1.4	3.0 \pm 2.6 143 \pm 117 (51 - 312)	2.5 \pm 1.8 120 \pm 41 (72 - 172)
Rifampin	AUC _{0-last} , $\mu\text{M}\cdot\text{hr}$		67.37 \pm 24.41	1.24 \pm 1.17
	C _{max} , μM		14.80 \pm 4.94	0.32 \pm 0.26

Values are Mean \pm SD, n=4.

Values in parentheses are range.

* Indicates statistically significant difference from control ($p < 0.05$).

**N/A = not available.

TABLE 3

Pharmacokinetic parameters of MDZ and 1'-OH MDZ following intra-hepatic portal vein administration of MDZ (0.15 mg/kg/hr for 4 hr) to monkeys dosed with vehicle or compound A (6 mg/kg i.v.)

Compound measured	Parameters	Vehicle Control	Compound A 6 mg/kg i.v.
MDZ	AUC _{0-last} , $\mu\text{M}\cdot\text{hr}$	0.037 \pm 0.02	0.304 \pm 0.17* <i>% of control</i> 1062 \pm 753 (483 - 2072)
	AUC _{0-inf} , $\mu\text{M}\cdot\text{hr}$	0.040 \pm 0.02	0.328 \pm 0.21* 1022 \pm 695 (478 - 1943)
	t _{1/2} , hr	0.9 \pm 0.4	1.0 \pm 0.5 119 \pm 20 (95 - 136)
1'-OH MDZ	AUC _{0-inf} , $\mu\text{M}\cdot\text{hr}$	0.063 \pm 0.04	0.170 \pm 0.09* 439 \pm 350 (101 - 889)
1'-OH MDZ/MDZ Ratio	AUC _{0-inf}	1.6 \pm 0.7	0.55 \pm 0.07* 39 \pm 12 (21 - 46)
Compound A	AUC _{0-last} , $\mu\text{M}\cdot\text{hr}$		6.73 \pm 1.61
	C _{max} , μM		1.88 \pm 0.35

Values are Mean \pm SD, n=4.

Values in parentheses are range.

*Indicates statistically significant difference from control ($p < 0.05$).

Fig. 1

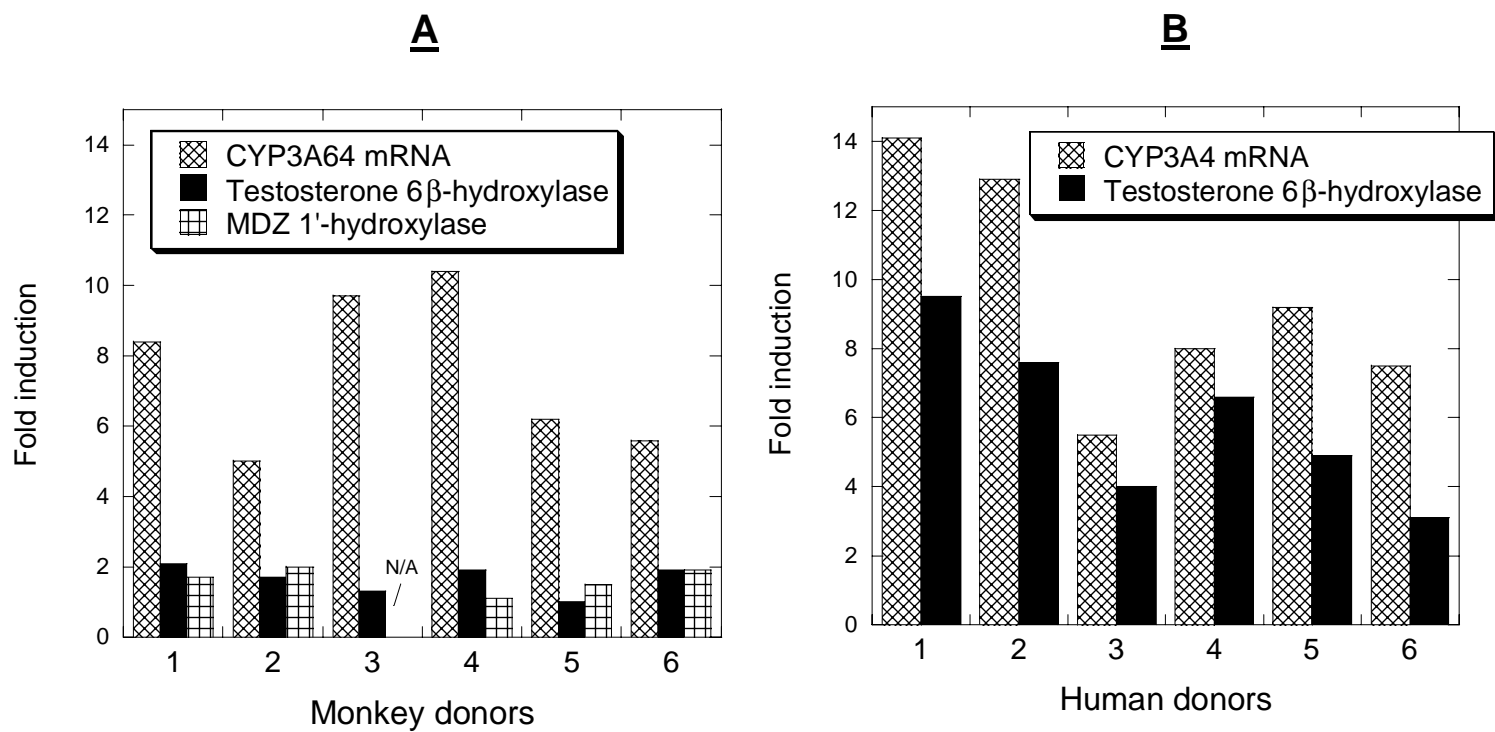


Fig. 2

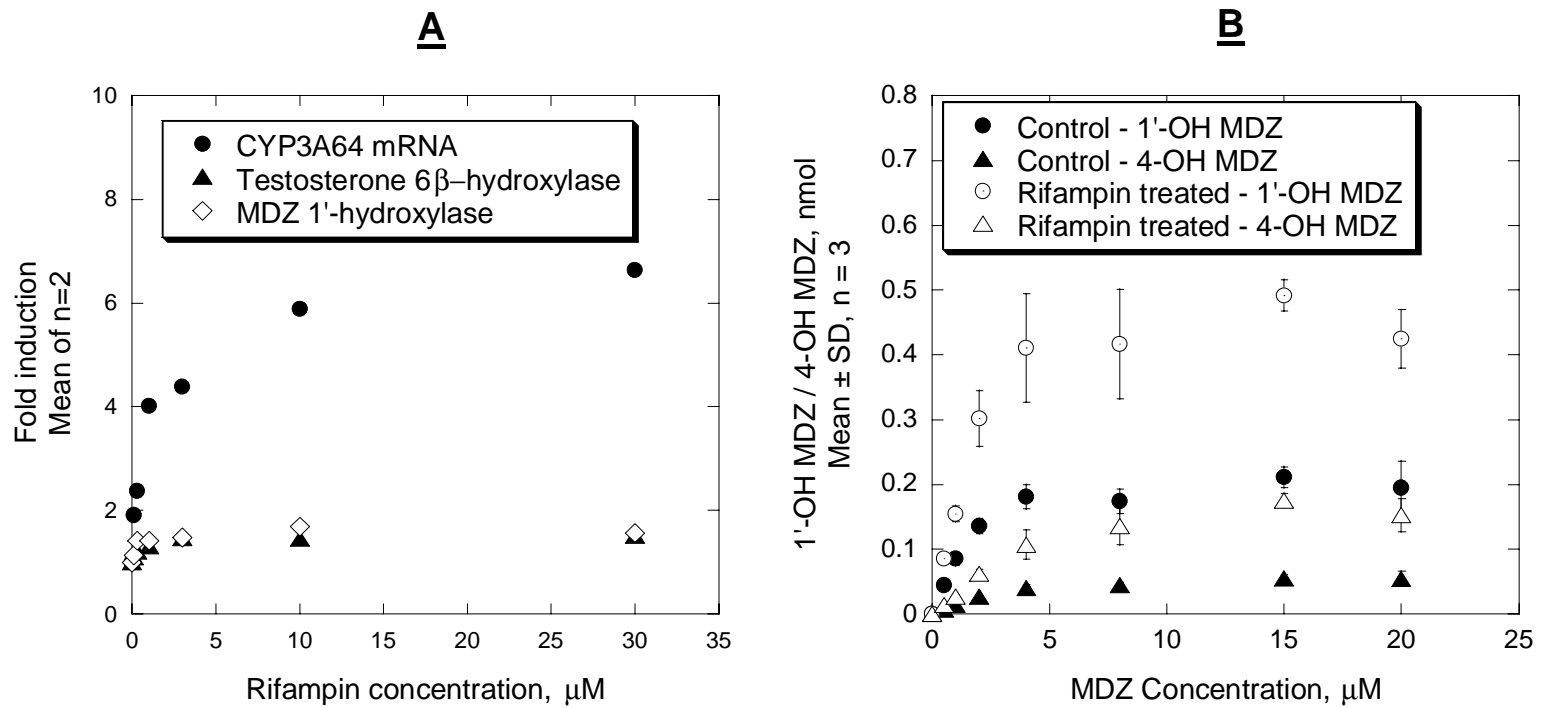


Fig. 3

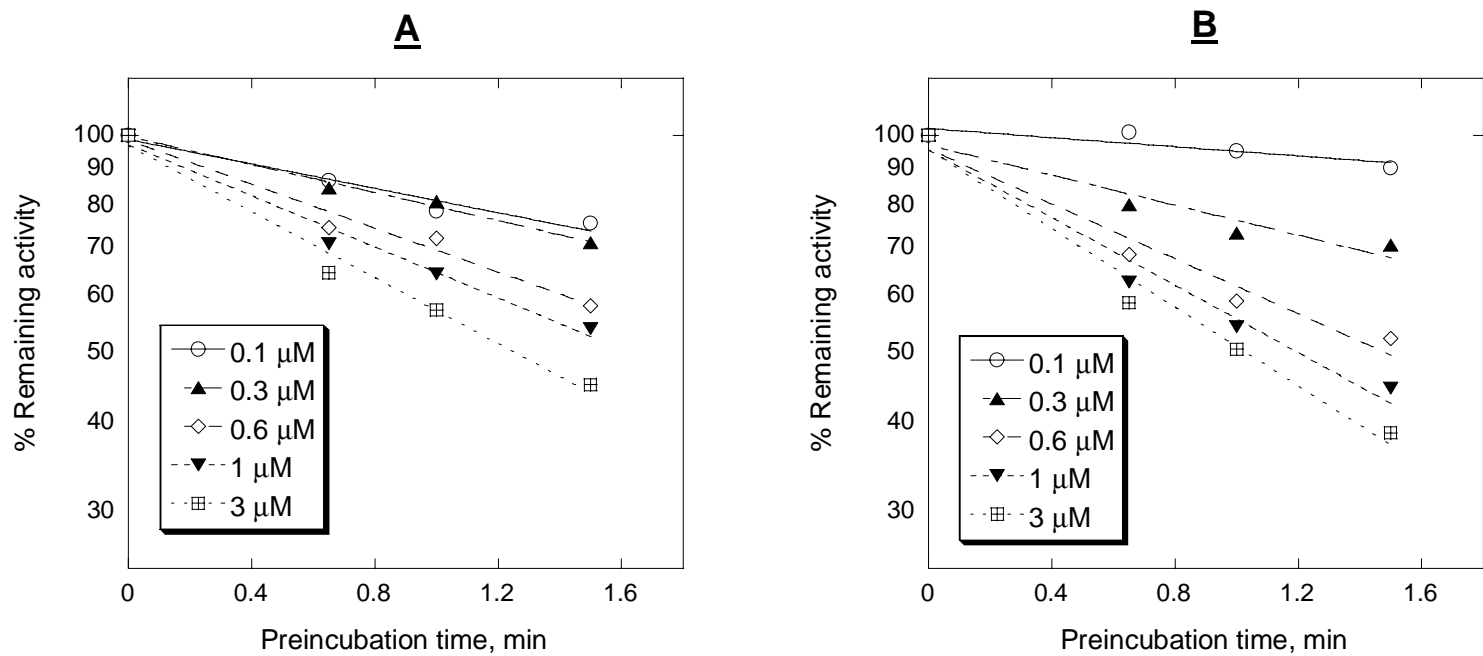


Fig. 4

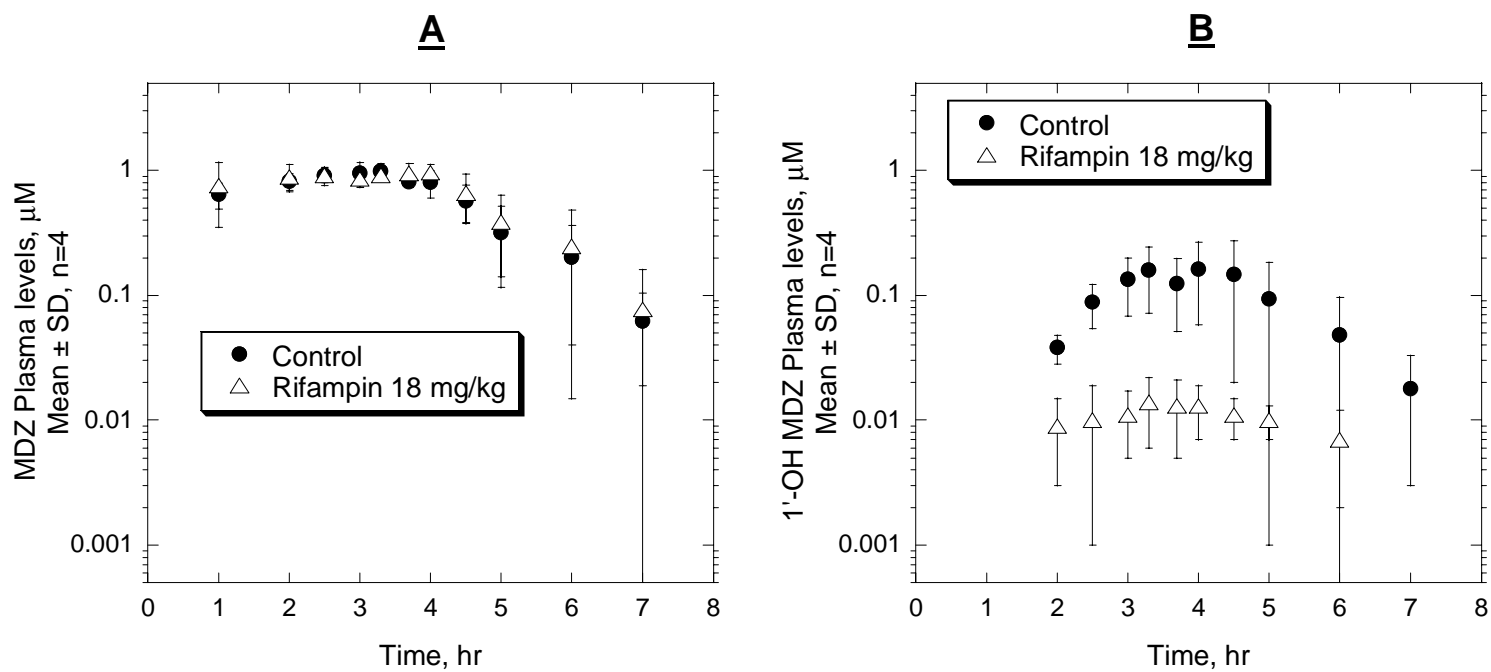


Fig. 5

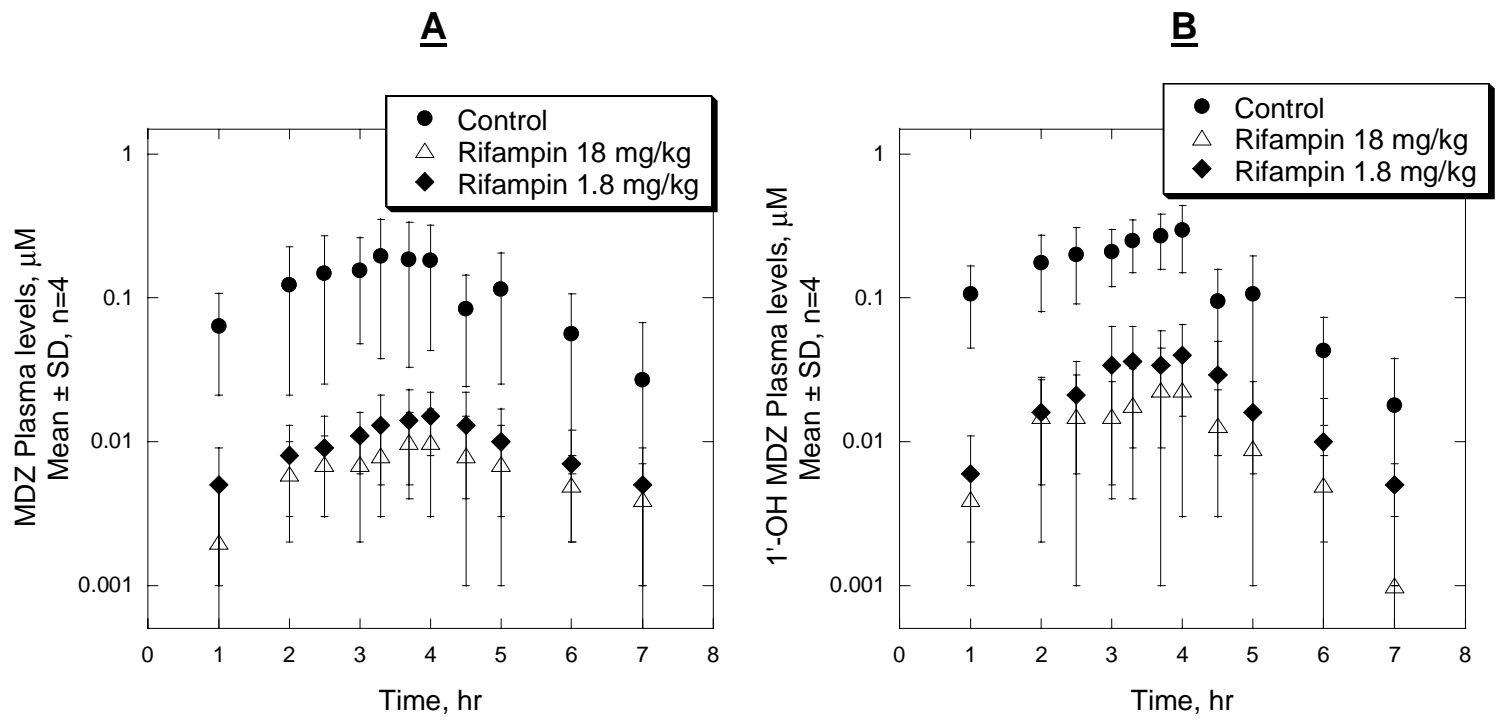


Fig. 6

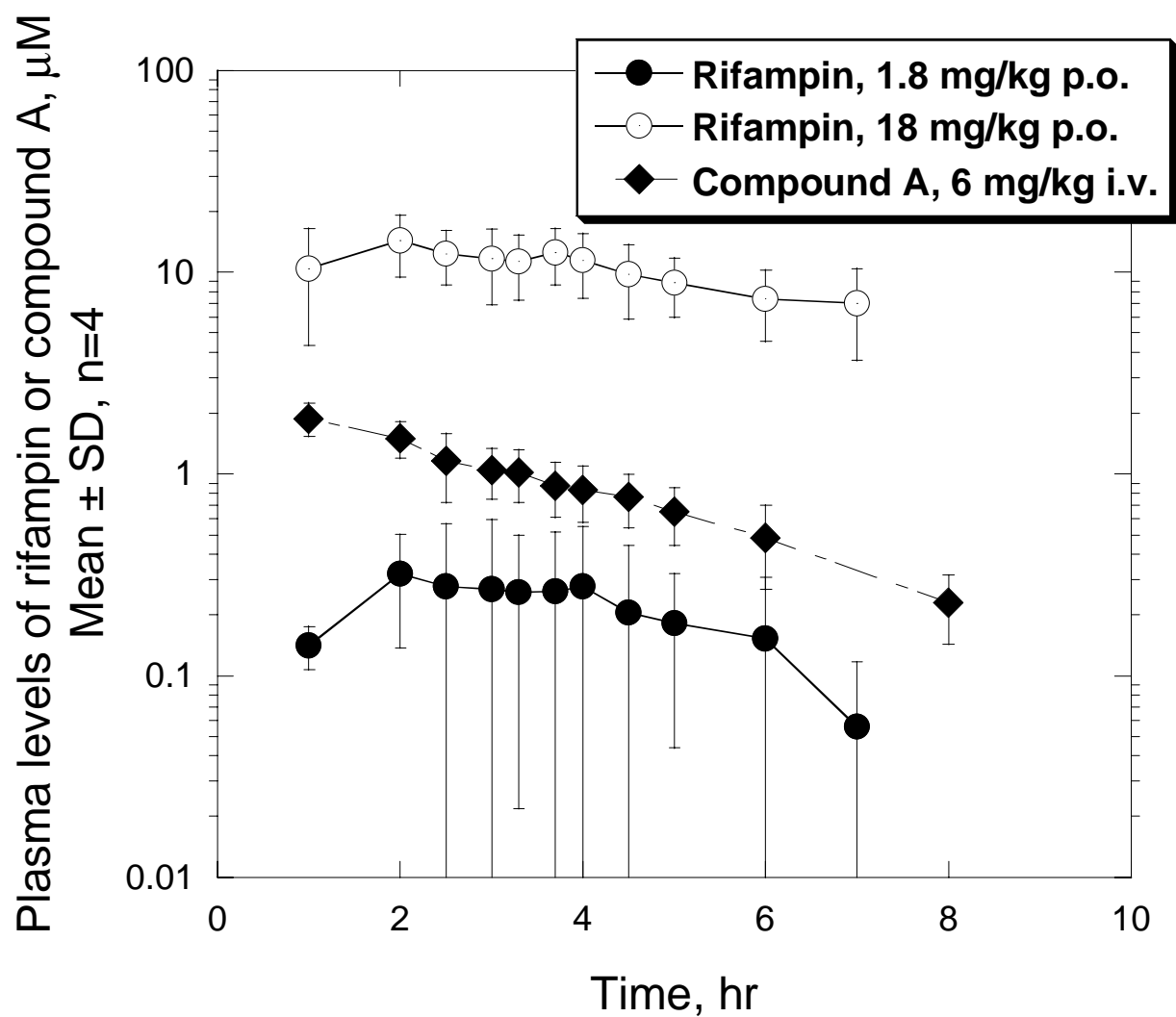


Fig. 7

