Inhibition of CYP3A by Erythromycin: In Vitro-In Vivo Correlation in Rats

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Received May 10, 2009; accepted September 28, 2009

ABSTRACT:

The prediction of in vivo drug-drug interactions from in vitro enzyme inhibition parameters remains challenging, particularly when time-dependent inhibition occurs. This study was designed to examine the accuracy of in vitro-derived parameters for the prediction of inhibition of CYP3A by erythromycin (ERY). Chronically cannulated rats were used to estimate the reduction in in vivo and in vitro intrinsic clearance (Clint) of midazolam (MDZ) after single and multiple doses of ERY; in vitro recovery of Clint was determined at 1, 2, 3, and 4 days after discontinuation of ERY. Enzyme inhibition parameters (kinact, Ki, and Kd) of ERY were estimated in vitro by using untreated rat liver microsomes. In vivo enzyme kinetic analysis indicated that single and multiple doses of ERY (150 mg/kg i.v. infusion over 4 h) reduced MDZ Clint by reversible and irreversible mechanisms, respectively. CYP3A inactivation after multiple doses of ERY treatment reflected metabolic intermediate complex formation without a significant change in hepatic CYP3A2 mRNA. A physiologically based pharmacokinetic model of the interaction between ERY and MDZ predicted a 2.6-fold decrease in CYP3A activity after repeated ERY treatment using in vitro-estimated enzyme inhibition parameters and in vivo degradation half-life of the enzyme (20 ± 6 h). The observed -fold decreases were 2.3-fold and 2.1-fold for the in vitro-estimated CYP3A activity and the in vivo Clint, respectively. This study demonstrates that in vivo DDIs are predictable from in vitro data when the appropriate model and parameter estimates are available.

The prediction of in vivo drug-drug interactions (DDIs) from in vitro data has met with limited success, and the general applicability of such predictions is unclear (von Molnke et al., 1998; Wang et al., 2004; Obach et al., 2005, 2007). As drugs capable of mechanism-based inhibition (MBI) have emerged as clinically important CYP3A inhibitors (Zhou et al., 2004), the uncertainty in predictive accuracy has increased. Approaches to predicting MBI-based DDIs vary from a relatively simple method using a single-inhibitor concentration to more complicated physiologically based pharmacokinetic (PBPK) models that consider the change of inhibitor and substrate concentrations with time (Mayhew et al., 2000; Ito et al., 2003; Wang et al., 2004; Obach et al., 2007). Changes in the amount of active enzyme in the presence of a mechanism-based inhibitor are estimated by using the in vitro enzyme inactivation parameters, Ki and kinact, which can be used to predict the increase in the in vivo exposure of the substrate. These predictions often suffer from poor characterization of substrate and inhibitor properties in vitro and/or in vivo and from uncertainties in predictive model parameter values. For example, DDIs may reflect simultaneous reversible inhibition, irreversible inhibition, and induction at multiple sites of enzyme expression, but the relative contribution of each type of interaction in vivo may be difficult to define. PBPK models provide a manageable path forward for addressing these potentially complex DDIs and have the important advantage of accommodating the mutual, nonlinear interactions between inhibitors and substrates. However, these approaches are based on the often untested assumption that mechanisms and potencies of interactions can be readily translated from in vitro systems to the whole animal.

The most common locus of DDIs is the cytochrome P450 (P450) family of enzymes and the CYP3A subfamily in particular. Midazolam (MDZ) is the most common substrate used to characterize CYP3A activity in humans and rats because of its high CYP3A selectivity and its lack of significant interaction with cell membrane transporters (Franke et al., 2008). CYP3A2 and CYP2C11 are the major P450 isoforms found in male rat liver (Shaw et al., 2002), and MDZ is almost exclusively metabolized by CYP3A2 to form primarily 4-OH MDZ (Shaw et al., 2002; Uhing et al., 2004).

ABBREVIATIONS: DDI, drug-drug interaction; MBI, mechanism-based inhibition; PBPK, physiologically based pharmacokinetic modeling; P450, cytochrome P450; MDZ, midazolam; ERY, erythromycin; MIC, metabolic intermediate complex; Clint, intrinsic clearance; AUC, area under the curve; RLM, rat liver microsome; PCR, polymerase chain reaction; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.
The effect of erythromycin (ERY) administration on CYP3A activity is complex and potentially reflects the net effect of enzyme induction, reversible inhibition, and irreversible inhibition (Danan et al., 1981; Amacher et al., 1991; Yamano et al., 2000). ERY, a tertiary amine, forms a stable metabolic intermediate complex (MIC) in vivo, which displays a characteristic Soret peak approximately 456 nm, and inactivates CYP3A (Pershing and Franklin, 1982). Approximately 40% of total P450 forms MIC after daily oral administration of 800 mg of ERY to humans (Amacher et al., 1991). It is interesting to note that no complex could be detected in human liver microsomes isolated after a single oral dose of ERY (Danan et al., 1981; Yamano et al., 2000). It has been suggested that chronic ERY administration is capable of inducing P450s. For example, repeated oral administration of ERY, 2 mmol/kg daily for 4 days in humans increased hepatic microsomal protein concentration as well as ERY N-demethylase activity (Danan et al., 1981). In contrast, several other studies have reported no induction after chronic ERY treatment in rats (Yamano et al., 2000; Takedomi et al., 2001).

In this study, we examined the interaction between MDZ and ERY by using a previously validated, physiologically stable chronically cannulated rat model that allows these complex interactions to be quantified in vivo (Uhing et al., 2004; Quinney et al., 2008). The in vitro parameters characterizing reversible and irreversible components of CYP3A inhibition by ERY were determined by using hepatic microsomes from rats treated with ERY. Finally, we developed a physiological model for quantitatively predicting the magnitude of the in vivo changes in intrinsic clearance of MDZ based upon in vitro-estimated enzyme inhibition parameters.

Materials and Methods

Chemicals. MDZ (Versed; Roche Pharma Inc., Basel, Switzerland) was diluted in sterile saline to the desired concentrations before intravenous administration. Sterile erythromycin lactobionate (Erythromycin; Abbott Laboratories, Abbott Park, IL) was reconstituted in 5% dextrose injection (Uhing et al., 2004).

Animals. Male Sprague-Dawley rats weighing 325 to 350 g were obtained from Charles River Laboratories, Inc. (Wilmington, MA). Rats were housed individually in standard cages and were allowed free access to water and food pellets. Animals were maintained under constant temperature and humidity and a daily 14/10-h light/dark cycle. The drug disposition studies were performed in free-moving, anesthetized animals. They remained calm in their cages where their behavior was placid during the experimental procedures of drug administration and blood withdrawal. The experimental protocol was approved by the Institutional Animal Care and Use Committee of Rush Medical Center.

Placement of the Catheters. Rats were anesthetized with a mixture of ketamine (60 mg/kg) and xylazine (5 mg/kg) administered intramuscularly. Catheters were placed in the aorta, inferior vena cava, and hepatic vein as described previously (Uhing et al., 2004).

In Vivo Pharmacokinetics. The disposition of MDZ after intravenous administration was used as a surrogate for in vivo hepatic CYP3A activity (Uhing et al., 2004). Animals were divided into four groups, as described below, and received single or multiple treatments of ERY designed to reversibly or irreversibly inhibit CYP3A activity. The pharmacokinetics of MDZ were characterized in all animals after intravenous administration of MDZ. All experiments began 4 days after catheter implantation to allow animals to completely recover from the effect of surgery and anesthesia.

Irreversible CYP3A inhibition was studied in the first group of animals (n = 9) after multiple doses of ERY. MDZ (5 mg dissolved in 1 ml of normal saline) was administered via the inferior vena cava by a constant infusion over 2 min. Simultaneous blood samples were obtained from the aorta and hepatic vein (0.2 ml) at 5, 7.5, 10, 15, 20, 30, 45, 60, 90, 120, and 180 min after the start of infusion for the determination of baseline MDZ clearance. One day later and daily for 4 successive days, 150 mg of ERY was administered by infusion over 6 h via the inferior vena cava. Hepatic vein blood samples were obtained at 0, 3.5, 4.5, and 5.5 h after the start of the infusion on each day and assayed for plasma ERY concentrations. At the midpoint of the final ERY infusion, i.e., at 3 h, on day 4, MDZ disposition was determined as described previously. Then, on days 1, 2, and 3 after the last dose of ERY, animals (n = 3) were sacrificed and their livers obtained to quantify CYP3A activity in vitro. A second group of animals (n = 3) were sacrificed on the 4th day of ERY treatment, 3 h after the start of the final ERY infusion to obtain control livers not exposed to MDZ.

Competitive inhibition was examined in a third group of rats (n = 3). A single dose of ERY (150 mg of infused over 6 h) was administered intravenously via the inferior vena cava. MDZ (5 mg dissolved in 1 ml of normal saline) was administered at the midpoint of ERY infusion via the inferior vena cava by a constant infusion over 2 min. MDZ disposition was determined as described previously. Livers were obtained immediately upon discontinuation of ERY.

The final group of rats (n = 3) underwent sham surgery and served as controls. Rats received saline infusions identical to ERY administration, i.e., a 1-ml infusion via the inferior vena cava over 2 min for 4 days. Livers were removed at the end of saline infusion on day 4.

In all studies, after each blood sample withdrawal, animals were immediately transfused with an equal volume of blood obtained from another set of healthy, chronically catheterized male Sprague-Dawley rats. The hematocrit in all study animals remained stable over the time course of drug administration and blood sample withdrawal.

Sample Size Justification. A previous study indicated that the coefficient of variation of MDZ intrinsic clearance (CLint) was approximately 7.7% (Yamano et al., 2000). With this coefficient of variation and three rats in each group, there is 80% power to detect a difference of 25 ml/min/kg in the CLint of MDZ, at a significance level of 0.05.

The MDZ PK Model. A compartmental pharmacokinetic model was developed to describe MDZ disposition, as illustrated in Fig. 1. The differential equations describing MDZ concentrations in each compartment are shown below and account for mass balance.

\[
\begin{align*}
\frac{dC_A}{dt} &= R_0 - \frac{Q_{HI} \times (C_{HI} - C_A)}{V_{HI}} - k_{12} \times C_A + k_{21} \times C_{PER} - \frac{V_{PER} \times C_{PER}}{V_C} \\
\frac{dC_{HV}}{dt} &= \frac{Q_{HI} \times (C_{HI} - C_{HV})}{V_{HI}} - \frac{V_{max} \times C_{HV}}{K_m + C_{HV}} \\
\frac{dC_{PER}}{dt} &= k_{12} \times C_A - k_{21} \times C_{PER}
\end{align*}
\]

where \(C_A, \ C_{HI}, \ \text{and} \ C_{PER} \) are concentrations in the central compartment (i.e., aorta), the liver (i.e., hepatic vein), and the peripheral compartment, respectively. This approach is based on the well stirred model (Rowland et al., 1973) and assumes that drug concentration in the liver is in instantaneous equilibrium with drug concentration in the hepatic vein. \(R_0\) is the infusion rate of MDZ. \(V_C\), \(V_{PER}\), and \(V_{HI}\) are the volume of central compartment, peripheral compartment, and liver, respectively. \(Q_{HI}\) is liver blood flow. \(k_{12}\) and \(k_{21}\) are the distribution rate constants for drug transfer between central and peripheral compartments. \(V_{max}\) and \(K_m\) are the maximal rate of metabolism and the Michaelis-Menten constant for MDZ.

To test whether the base model (eqs. 1–3) could be reduced to better fit the data, the macro-constants A, B, α, and β for a classic, two-compartment intravenous infusion pharmacokinetic model were obtained by curve-stripping of the individual MDZ blood concentration-time data. \(V_C\) and \(V_{PER}\) were calculated by using equations listed in the Appendix (eqs. 12–17). This compartmental analysis revealed that the values (mean ± S.D.) of volume of central and peripheral compartment were not significantly different based on paired Student’s t test: \(V_C\) = 526 ± 133 ml, \(V_{PER}\) = 478 ± 166 ml (n = 14, p = 0.44). Therefore, eq. 1 and 3 in the MDZ PK model were simplified to eqs. 1’ and 3’, which improved the precision in the estimation of the model parameters.

\[
\begin{align*}
\frac{dC_A}{dt} &= R_0 - \frac{Q_{HI} \times (C_{HI} - C_A)}{V_{HI}} - k_{12} \times C_A + k_{21} \times C_{PER} \\
\frac{dC_{PER}}{dt} &= k_{12} \times C_A - k_{21} \times C_{PER}
\end{align*}
\]
In both the initial and reduced models, $R_d$, $Q_{HA}$, and $V_H$ were treated as constants. $V_H$ was calculated from liver weight assuming a density of liver tissue of approximately 1 g/ml. $Q_{HA}$ was estimated with baseline MDZ concentrations by using the after equations for each rat.

$$\text{Metabolic Rate} = \frac{Q_{HA}}{V_H} \times (C_A - C_{IV})$$

(4)

$$Q_{HA} = \frac{CL_{HA}}{ER}$$

(5)

$$CL_{HA} = \text{Dose/AUC}_{A}$$

(6)

$$ER = \frac{(C_A - C_{IV})}{C_A}$$

(7)

where $CL_{HA}$ and $ER$ are hepatic clearance and hepatic extraction ratio, respectively. $AUC_{A}$ is the AUC calculated from aortic concentration-time data.

Plasma MDZ concentrations were converted to blood concentrations based on a blood-to-plasma ratio of 0.7 (Takedomi et al., 2001). The aorta and hepatic vein concentration-time data of MDZ before and after ERY treatments were fitted simultaneously to the base and reduced models (eqs. 1, 2, and 3') for the estimation of $V_{max}$ and $K_{inact}$. Based on the goodness-of-fit criteria, the weighted sum of square of residuals (WSS2) of the reduced model was significantly smaller than that of the base model (WSS2′ = 0.23 ± 0.12 and 1.64 ± 0.95 for reduced and base models, respectively; $p < 0.05$). Therefore, the reduced model was adopted for the estimation of changes in $V_{max}$ and $K_{inact}$.

**In Vivo Enzyme Kinetic Analysis.** Based on the predicted aortic and hepatic vein concentration-time profiles of MDZ, the in vivo metabolic rates were calculated based on eqs. 4 to 7. The metabolic rates were plotted against the corresponding MDZ concentration in the hepatic vein, which was assumed to be equivalent to MDZ concentration in the hepatocytes, consistent with the assumptions of the well-stirred model (Rowland et al., 1973). The Lineweaver-Burk plot was used to examine the type of in vivo inhibition observed after single and multiple doses of ERY.

**Determination of Plasma Concentration of ERY and MDZ.** Plasma concentration of ERY, MDZ, and 4-OH MDZ were determined by using liquid chromatography/mass spectrometry methods as described previously (Belle et al., 2002; Lam et al., 2006). The plasma concentrations of MDZ and 4-OH MDZ were quantified by liquid chromatography/mass spectrometry. After the addition of the internal standard (desmethyl Diazepam; 30 µl of a 10 µg/ml solution; Sigma-Aldrich, St. Louis, MO), and 0.5 ml of a 1 mM sodium hydroxide/glycine buffer (pH 11.3) to 0.5 ml of plasma, the samples were extracted with 5 ml of cyclohexane/ethyl acetate (50:50, v/v) and the organic layer evaporated to dryness under nitrogen. The dried sample extract was reconstituted with 120 µl of mobile phase (acetone/trimethanol/10 mM ammonium acetate (pH = 7.6), 40:20:40 v/v/v), and 10 µl was injected onto the column (C18 Luna 3 µm 2.0 100 mm; Phenomenex, Torrance, CA). The column was eluted isocratically with mobile phase at a flow rate of 0.2 ml/min. The effluent was delivered to a mass spectrometer (Navigator; Thermo Fisher Scientific, Waltham, MA). The electrospray ionization probe was run in the positive ion mode with probe temperatures of 400°C. MDZ and 4-OH MDZ were detected in the selected ion recording mode at m/z of 326 and 342, respectively. The limit of quantification in plasma was 200 pg for MDZ and 4-OH MDZ.

**Determination of Microsomal Protein Concentration and P450 Complex Formation.** Rat livers were weighed, and the rat liver microsomes (RLMs) were prepared by the method of differential centrifugation (Franklin and Estabrook, 1971). The total microsomal protein concentrations were determined by the method of Lowry (Lowry et al., 1951). The concentration of uncomplexed P450s, complexed P450s, and total P450s were measured independently as described previously (Omura and Sato, 1962; Franklin, 1991). In particular, for the determination of total P450, 10 µl of potassium ferricyanide (5 mM) was first added to a 1-mL solution containing 1 mg of microsomal protein, 100 mM sodium phosphate buffer (pH = 7.4), and 5 mM magnesium chloride to breakdown P450s that were present as MIC. After decomplexation, the procedure used for the measurement of free P450s was followed to obtain total P450 content.

**Determination of CYP3A Activity in Rat Liver Microsomes.** The CYP3A activity in microsomes from the livers obtained at baseline, after a single dose of ERY, at the end of multiple doses of ERY, and on the days after the discontinuation of ERY was quantified in vitro by using 4-OH MDZ formation as the probe reaction. In brief, 0.25 mg of microsomal protein was incubated with MDZ at a saturating concentration of 500 µM in sodium phosphate buffer (0.1 M, pH = 7.4) with a final incubation volume of 1 ml. NADPH (1 mM final concentration) was added and the microsomes were incubated at 37°C for 5 min. The enzyme reaction was terminated by adding 1 ml of ice-cold acetonitrile. All experiments were performed in triplicate.

**Isolation and Measurement of CYP3A2 mRNA.** Total RNA was isolated from rat livers by using the RNeasy mini kit (Qiagen Inc., Valencia, CA), according to the manufacturer's instructions. RNA yield was determined by spectrophotometry (Beckman DU 640; Beckman Coulter, Inc., Fullerton, CA), and quality was assessed by the 260/280 nm ratio. cDNA was synthesized from 1 g of total RNA in a 20-µl mixture using random hexamers and avian myeloblastosis virus reverse transcriptase (Promega, Madison, WI). A real-time polymerase chain reaction (PCR) method was used to determine the amount of hepatic CYP3A2 mRNA based on a previously described method (Ronis et al., 2006). Primers specific to CYP3A2 transcripts were purchased from Integrated DNA Technologies, Inc. (Coraville, IA). Rat CYP3A2-specific forward and reverse primers were 5′-TCT CTA CCG ATT GGA ACC CAT AG-3′ and 5′-TTG TAG TAA TTC AGC ACA GTG CCT AA-3′. The CYP3A2 expression was normalized to that of the housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH). The forward and reverse primers for GAPDH were 5′-TGA TGG TGC TGC TG A-3′ and 5′-TTG ATT TGG TCC TTC AGC ACA CCA CAC ATC ACA-3′, respectively. Melt-curve analysis was used to ensure that the expected PCR products were being generated continuously and reproducibly in
where plots were constructed to differentiate modes of inhibition.

Reversible and Irreversible Inhibition Parameter Estimation for ERY with RLMs. To estimate the reversible inhibition constant $K_R$, MDZ (10–200 μM) and ERY (25–300 μM) were incubated with RLMs (0.25 mg) in a 50-μl reaction mixture with ERY at 2.5, 10, 25, and 100 μM in the presence of NADPH (1 mM) at 37°C for 0, 1, 2, and 5 min. Then, 950 μl of incubation mixture containing MDZ and 1 mM NADPH in 0.1 M sodium phosphate buffer were transferred into the microcentrifuge tube (to achieve a final MDZ concentration of 300 μM) and further incubated at 37°C for 5 min.

The reversible inhibition constant $K_R$ was estimated by fitting the appropriate inhibition models (competitive, noncompetitive, or uncompetitive) to the 4-OH MDZ formation rate versus MDZ concentration data by using nonlinear regression (WinNonlin 4.0; Pharsight, Mountain View, CA). Lineweaver-Burk plots were constructed to differentiate modes of inhibition.

To estimate the inactivation constants, the natural logarithm of the percent- age of the remaining activity was plotted against preincubation time. The observed inactivation rate constants ($k_{inact}$) were determined from the slopes of the initial linear decline in activity. The parameters $k_{inact}$ and $K_I$ were obtained from simultaneous fitting of the data of the percentage of the remaining activity versus the preincubation time at all inhibitor concentrations by using nonlinear regression (WinNonlin 4.0; Pharsight) according to eqs. 8 and 9:

$$E_0 = E_0 \cdot e^{-k_{inact} \cdot t}$$

$$k_{obs} = \frac{k_{max} \cdot I}{K_I + I}$$

where $E_0$ and $E_t$ are enzyme activity at time 0 and $t$, respectively, $k_{obs}$ is inactivation rate constant, $k_{inact}$ is the rate constant that defines the maximal rate of inactive enzyme formation, $I$ is the initial concentration of the inhibitor, and $K_I$ is the inhibitor concentration when $k_{obs} = k_{max}/2$.

Prediction of In Vivo CYP3A Inhibition by Erythromycin. Prediction using a single-inhibitor concentration. A simple algebraic equation was used to predict the -fold decease in $CL_{int}$ of MDZ after multiple doses of ERY treatment as shown in eq. 10 (Wang et al., 2004).

$$CL_{int}' = \frac{f_m}{1 + k_{obs} \cdot I} + (1 - f_m)$$

where $f_m$ is the fraction of the total hepatic elimination that is due to the CYP3A pathway in the absence of the inhibitor, $I$ is the unbound inhibitor concentration. A defined value of $f_m$, for MDZ could not be obtained from the literature, thus a value of 0.9 was assumed for $f_m$, for the prediction based on a study that suggested MDZ was almost “exclusively” metabolized by CYP3A to form 4-OH MDZ in rats (Shaw et al., 2002). Because MDZ was given 3 h into ERY infusion, a time-averaged ERY concentration from 3 to 5.5 h after the initiation of ERY infusion was calculated and considered to best reflect the inhibitor exposure. Fraction of unbound ERY in rat plasma is 0.15 (Lam et al., 2006). The values of enzyme parameters ($k_{max}$, $K_I$ and $k_{obs}$) were used for eq. 10 were the same as those used for the interaction model.

The values of four key model parameters ($f_m$, $k_{max}$, $K_I$ and $K_{inact}$) were varied 10-fold ($f_m$ varied from 0.8 to 0.99 to be more realistic for MDZ) within the simulation environment to rank order the importance of their influences on predicting ERY inhibition of MDZ clearance.

Prediction Using a PBPK Approach. To predict the change in the amount of CYP3A in the liver in response to ERY treatment, an interaction model was developed as depicted in Fig. 1. A multicompartment model was developed for ERY based on the reported pharmacokinetic and physiological parameters in rats. In this model, $V_C$, $V_{pre}$, and $V_{hep}$ are the volume of central, peripheral, and liver compartment, respectively. $k_{i2}$ and $k_{2i}$ are the rate constants for distribution between the central and peripheral spaces. CYP3A enzyme pool in the liver was modeled as a separate compartment (Fig. 1, Enzyme Model) based on eq. 11 (Zhang et al., 2008).

$$\frac{dE_t}{dt} = k_{deg} \cdot E_t - k_{act} \cdot E_t - \frac{k_{max} \cdot I_{obs}}{K_I + I_{obs}} \cdot E_t$$

where $E_t$ and $E_0$ are enzyme activity at time 0 and $t$, respectively, $k_{deg}$ is CYP3A degradation rate constant, and $I_{obs}$ is the unbound inhibitor concentration at time $t$.

After ERY administration, the unbound concentration of ERY in the liver ($I_{obs}$) together with the inactivation parameters ($k_{max}$ and $K_I$) determine the inactivation rate constant, which, in turn, determines the change in the amount of CYP3A in the liver with time.

The parameters used for the ERY model and the enzyme model are listed in Table 1. Simulations were performed by using Pharsight Trial Simulator (Pharsight) to predict the time course of ERY and the corresponding change in CYP3A activity under the scenario of ERY 150 mg of intravenous infusion for 6 h once a day for 4 days. The result was compared with the observed ERY concentrations and the decrease in CYP3A activity observed both in vivo and in vitro.

Results

MDZ aortic and hepatic vein blood concentration-time data were simultaneously fit to a nonlinear 2-compartment model depicted in Fig. 1 (Drug Model). The model provided a good fit to the data obtained in the absence of ERY, after multiple doses of ERY (150 mg by infusion over 6 h for 4 days), and after a single dose of ERY (150 mg infused over 6 h) (Fig. 2). MDZ concentrations in both the aorta and hepatic vein were elevated after single- and multiple-dose ERY treatment indicating significant inhibition of MDZ elimination. The difference between the fitted lines of aorta and hepatic vein is equal to the rate of hepatic extraction of midazolam (eq. 7). The intrinsic clearance ($V_{Cl}/K_{inact}$) of MDZ, estimated from the fitting of the blood concentration-time data, was reduced by ERY by 1.5 ± 0.7-fold and 2.3 ± 1.4-fold for the single and multiple doses of ERY treatment, respectively (Table 2).

Figure 3 shows the relationship between the estimated in vivo rates of MDZ metabolism, determined from the postdistributional difference in aortic and hepatic vein blood concentrations (eqs. 4–7), and substrate concentration; classic Michaelis-Menten relationships were observed. The in vivo $V_{max}$ was reduced from 136.9 to 71.4 μg/min after multiple ERY doses, as reflected in the higher $y$-intercept in double reciprocal plot (Fig. 3, A and B). Single-dose ERY did not affect the maximal MDZ metabolic rate but increased the in vivo $K_m$ from 1.4 to 3.1 μg/ml; this result is reflected in the greater negative $x$-intercept in the double reciprocal plot (Fig. 3, C and D).

<table>
<thead>
<tr>
<th>TABLE 1</th>
<th>Model parameters for ERY and CYP3A used in the simulation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Parameters</td>
<td>Values</td>
</tr>
<tr>
<td>$V_C$ (ml)</td>
<td>200</td>
</tr>
<tr>
<td>$V_{pre}$ (ml)</td>
<td>400</td>
</tr>
<tr>
<td>$CL_{hep}$ (l/h)</td>
<td>0.05</td>
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<tr>
<td>$CL_{int}$ (l/h)</td>
<td>0.54</td>
</tr>
<tr>
<td>$f_m$</td>
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<tr>
<td>Physiological parameters</td>
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<tr>
<td>$Q_{sm}$ (ml/min)</td>
<td>22*</td>
</tr>
<tr>
<td>$V_{sm}$ (ml)</td>
<td>10*</td>
</tr>
<tr>
<td>$k_{deg}$ (h$^{-1}$)</td>
<td>0.0348</td>
</tr>
<tr>
<td>Enzyme inhibition parameters</td>
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<tr>
<td>$K_I$ (μM)</td>
<td>117</td>
</tr>
<tr>
<td>$k_{inact}$ (min$^{-1}$)</td>
<td>0.04</td>
</tr>
<tr>
<td>$K_{inact}$ (μM)</td>
<td>15.1</td>
</tr>
</tbody>
</table>

* $Q_{sm}$ and $V_{sm}$ were calculated for individual rat as described in the text under The MDZ PK Model. The mean values were used for the simulation.
The effect of ERY dosing in vivo on the in vitro hepatic CYP3A activity, determined as maximal rate of MDZ 4-hydroxylation, is shown in Fig. 4. There was no significant difference in CYP3A concentrations between the control rats and the rats treated with a single dose of ERY ($p = 0.06$). However, multiple doses of ERY significantly decreased CYP3A activity (2.1 ± 1.2-fold, $p < 0.01$) compared with controls. CYP3A activity recovered gradually after the last dose of ERY and returned to the control values by day 3 ($p = 0.4$; Fig. 4).

The effect of ERY treatment on the microsomal P450 content is summarized in Table 3. The presence of MIC was not detected in liver microsomes from control rats or rats treated with a single dose of ERY, but it was readily detected after multiple doses of ERY. The microsomal MIC content declined upon discontinuation of ERY and

### Table 2

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control</th>
<th>Multiple-Dose ERY</th>
<th>Control</th>
<th>Single-Dose ERY</th>
</tr>
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<tbody>
<tr>
<td>$V_{max}$ (mg/ml/min)</td>
<td>142 ± 54</td>
<td>70.8 ± 31.3</td>
<td>130.6 ± 33.4</td>
<td>129.8 ± 35.9</td>
</tr>
<tr>
<td>$K_m$ (mg/ml)</td>
<td>1.3 ± 0.4</td>
<td>1.3 ± 0.6</td>
<td>1.5 ± 1.3</td>
<td>1.9 ± 1.5</td>
</tr>
<tr>
<td>$k_{in}$ (min$^{-1}$)</td>
<td>0.09 ± 0.04</td>
<td>0.09 ± 0.04</td>
<td>0.09 ± 0.04</td>
<td>0.09 ± 0.04</td>
</tr>
<tr>
<td>$k_{out}$ (min$^{-1}$)</td>
<td>0.08 ± 0.03</td>
<td>0.08 ± 0.03</td>
<td>0.10 ± 0.05</td>
<td>0.10 ± 0.05</td>
</tr>
<tr>
<td>$V_C$ (ml)</td>
<td>295.1 ± 49.3</td>
<td>295.1 ± 49.3</td>
<td>217.3 ± 58.1</td>
<td>217.3 ± 58.1</td>
</tr>
<tr>
<td>Fold decrease in $V_{max}/K_m$</td>
<td>2.3 ± 1.4</td>
<td>2.3 ± 1.4</td>
<td>1.5 ± 0.7</td>
<td>1.5 ± 0.7</td>
</tr>
</tbody>
</table>

$p$ value was calculated for $V_{max}/K_m$. $k_{in}$, $k_{out}$, and $V_C$ were assumed the same for the simultaneous fitting of MDZ concentrations before and after ERY treatment, assuming ERY has no effect on these parameters.
had essentially disappeared by 3 days after the discontinuation of ERY. Table 3 also indicates that the content of uncomplexed P450 was not significantly changed by the treatment with either single or multiple doses of ERY. However, there was a nonstatistically significant increase in the total P450 content after multiple doses of ERY (0.49 ± 0.08 versus 0.71 ± 0.18 nmol/mg protein). Furthermore, as shown in Fig. 4A, the formation of MIC after multiple doses of ERY was coupled with significant decrease \((p < 0.01)\) in CYP3A activity, and the decline in the content of MIC after the last administration of ERY was accompanied with gradual recovery in CYP3A activity. Based on the slope of the semilogarithmic plot of the time course of the elimination of MIC after the last dose of ERY treatment of 4 days, the elimination rate constant was estimated to be 0.0349 h\(^{-1}\) (Fig. 4B). This result is equivalent to the degradation rate constant, \(k_{\text{deg}}\) of CYP3A in rats, and it translates into a CYP3A4 half-life of 20 h.

To examine whether induction occurred after ERY treatment, the relative expression of CYP3A2 mRNA normalized to GAPDH in the liver samples was determined by using real-time reverse transcription-PCR. No significant changes were observed in CYP3A2 at the mRNA level after multiple or single dose of ERY treatments (data not shown). The potency of ERY as both a mechanism-based inhibitor and a competitive inhibitor of CYP3A was evaluated in vitro with the RLMs. Figure 5A shows time- and inhibitor concentration-dependent inhibition of CYP3A by ERY. Coincubation of ERY and MDZ for 3 min resulted in apparent competitive inhibition of CYP3A (Fig. 5B). The estimated values (mean ± S.D.) for \(k_{\text{inact}}, K_I,\) and \(K_i\) are 0.04 ± 0.02 min\(^{-1}\), 15.1 ± 3.2 \(\mu\)M, and 117 ± 21 \(\mu\)M, respectively. These values were used for the prediction of in vivo CYP3A inhibition resulting from ERY treatments.

The inhibition of MDZ intrinsic clearance by ERY was predicted by two methods. The first method incorporated the frequently used assumption of a static, time-averaged inhibitor concentration, as described by eq. 10. For an assumed \(f_{\text{in}}\) of MDZ of 0.95, the corresponding prediction was a 15.4-fold decrease in the CL\(_{\text{int}}\) of MDZ (Table 4). The second method of prediction used dynamic inhibitor concentration-time profile for the same ERY multiple-dosing regimen used in our in vivo experiments. The predicted ERY concentrations are shown in Fig. 6, top panel, and the corresponding inhibition of hepatic CYP3A, using eq. 11, is illustrated in Fig. 6, bottom panel. The observed ERY concentrations were determined in blood samples at 3.5, 4.5, and 5.5 h after the start of ERY infusion. The percentage...
of baseline CYP3A activity remaining at the time when MDZ was given for the determination of the in vivo CYP3A activity was predicted to be 35% as indicated by the arrow in Fig. 6, bottom panel. The corresponding increase in MDZ AUC is predicted to be 2.6-fold, assuming an \( f_m \) by CYP3A of 0.95 for MDZ (instantaneous value at the time MDZ was given). This observation time corresponds to the time when the livers were removed from rats in the second experimental group for the measurement of CYP3A activity in vitro.

The influence of each variable on the predicted AUC ratio is shown in Fig. 7. The solid vertical line depicts the mean of AUC ratio, which was predicted by using the parameter values listed in Table 4. The horizontal bars depict the range of predicted AUC ratios at parameter values that span a 10-fold range and contain the mean value of each parameter used in the predictions presented earlier. The most influ-

![Graph showing in vivo MIC and CYP3A activity](image)

**TABLE 3**

<table>
<thead>
<tr>
<th></th>
<th>Uncomplexed P450</th>
<th>Complexed P450</th>
<th>Total P450</th>
<th>Value*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.45 ± 0.08</td>
<td>N.D.</td>
<td>0.49 ± 0.08</td>
<td>NA</td>
</tr>
<tr>
<td>Multiple dose of ERY</td>
<td>0.42 ± 0.07</td>
<td>0.31 ± 0.11</td>
<td>0.71 ± 0.18</td>
<td>0.18</td>
</tr>
<tr>
<td>1 Day after</td>
<td>0.47 ± 0.08</td>
<td>0.17 ± 0.07</td>
<td>0.67 ± 0.11</td>
<td>0.13</td>
</tr>
<tr>
<td>2 Days after</td>
<td>0.57 ± 0.29</td>
<td>0.09 ± 0.13</td>
<td>0.75 ± 0.38</td>
<td>0.15</td>
</tr>
<tr>
<td>3 Days after</td>
<td>0.46 ± 0.04</td>
<td>0.05 ± 0.03</td>
<td>0.58 ± 0.03</td>
<td>0.21</td>
</tr>
<tr>
<td>Single dose of ERY</td>
<td>0.49 ± 0.03</td>
<td>N.D.</td>
<td>0.52 ± 0.03</td>
<td>0.32</td>
</tr>
</tbody>
</table>

N.D., not detected; NA, not applicable.

* Value was calculated for total P450.
ential variable after the ERY dosing was the fraction of metabolic clearance attributable to the inhibited pathway \((f_{\text{in}})\). The \(k_{\text{inact}}\) of ERY and CYP3A degradation rate constant \(k_{\text{deg}}\) are equally influential within the 10-fold limits.

**Discussion**

The prediction of in vivo DDIs based on in vitro P450 inhibition potency is routinely undertaken to understand the drug interaction potential of established drugs and new chemical entities (Clarke and Jeffrey, 2001; Obach et al., 2007; Zhang et al., 2009). However, there is considerable uncertainty in the fidelity of these predictions, especially when MBI is involved. In the current study, we predicted the in vivo consequence of MBI after acute and chronic administration by using a PBPK model that takes into consideration the temporal change in the inhibitor concentration, the time course of the change in the active CYP3A enzyme pool, and its impact on the disposition of both inhibitor and substrate. We successfully predicted the decrease in the in vivo \(\text{CL}_{\text{int}}\) of MDZ after ERY treatment, and we demonstrated the utility of trans-hepatic concentration-time data obtained with a rat model to identify the nature of enzyme inhibition kinetics in vivo.
Our study design takes advantage of a chronically cannulated rat model in which the animals are allowed to completely recover from the effects of surgery and anesthesia. Experiments are performed after the animals have returned to nonstressed, physiological baseline after the placement of catheters. In a previous study, the concentration-time curves of MDZ were different in rats studied immediately after regaining the righting reflex and those 3 days postsurgery; this difference was reflected by a 45% increase in systemic clearance and a 50% decrease in hepatic availability in the chronically catheterized rats compared with the rats from acute surgery group (Uhing et al., 2004). This acute surgical effect is clearly

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undesirable yet is often present when the rat is used to study in vitro-in vivo extrapolation of drug clearance and to test predictive DDI models. False conclusions regarding the accuracy of such predictions may be reached if these studies are performed under the acute surgical conditions, which can alter the in vivo clearance.

In this study, simultaneous sampling of MDZ blood concentrations from the abdominal aorta and hepatic vein enabled us to measure the rate of extraction across the liver at any point in time. For MDZ, a drug exclusively eliminated in the rat liver by CYP3A2-mediated metabolism (Shaw et al., 2002), this extraction rate represents the rate of hepatic metabolism. Our pharmacokinetic analysis assumes the well stirred model of hepatic elimination, and hence the unbound concentration of MDZ in the hepatic vein (equivalent to the unbound hepatocyte concentration) is the driving force for hepatic elimination (Rowland et al., 1973; Wilkinson, 1987). Consequently, we are able to construct in vivo analogs of Michaelis-Menten curves that relate the rate of MDZ metabolism to the concentration of MDZ in hepatocyte (Fig. 3). In the presence of an inhibitor, this approach allows the mechanism of inhibition in vivo and the potency of the inhibitor to be determined (Fig. 3). In this first application of this approach, we characterized the in vivo inhibition profile of the well characterized CYP3A inhibitor, ERY. Our data indicate that in the rat, ERY is a weak competitive inhibitor of CYP3A after a single dose but a modestly potent “apparent noncompetitive” inhibitor after repeated dosing (Fig. 3). This transition from a competitive to an apparent noncompetitive mechanism of inhibition is precisely what would be expected for a mechanism-based inhibitor such as ERY. At a given point in time, noncompetitive inhibition and mechanism-based inhibition, both characterized by reduced $V_{\text{max}}$, cannot be distinguished.

In our novel approach, these mechanisms are resolved by the distinct temporal pattern of inhibition observed. This conclusion was consistent with the significant amount of MIC formed in the livers treated with multiple doses of ERY and lack of MIC formation in the control livers and livers obtained after the treatment with a single dose of ERY (Table 3; Fig. 4A).

The MDZ concentration-time data obtained from simultaneously sampling from the abdominal aorta and hepatic vein allows the estimation of hepatic intrinsic clearance directly from the in vivo $V_{\text{max}}$ and $K_m$ values (Rowland et al., 1973; Wilkinson, 1987). The intrinsic clearance estimates provided valuable information on the “true” change in the CYP3A activity in vivo and enabled comparison of the extent of change between the in vivo and in vitro settings. In the current study, after chronic ERY treatment, the intrinsic clearance estimated by simultaneously fitting the aorta and hepatic vein concentration-time data of MDZ was decreased approximately by 2.3-fold (Fig. 2; Table 2); the CYP3A activity in those livers determined in vitro by 4-hydroxy MDZ formation was reduced by 2.1-fold (Fig. 4A). These results suggest that the extent of CYP3A inhibition by ERY, independently estimated by using the in vitro and in vivo systems, was in good agreement. Furthermore, there was a 1.5-fold decrease in the in vivo intrinsic clearance, but no significant change was measured in the in vitro-estimated CYP3A activity after single dose of ERY administration (Table 2; Fig. 4A). This result is consistent with the hypothesis that treatment with a single dose of ERY inhibits CYP3A in a competitive manner and exerts no effect on the enzyme activity measured in vitro. These data are consistent with previous studies demonstrating that ERY inhibits CYP3A through two mechanisms, namely a quasi-irreversible formation of metabolic intermediate complexes (Lindstrom et al., 1993) and by competitive inhibition. With respect to competitive inhibition by ERY, Yamano et al. (2000) demonstrated an increase in MDZ AUC without MIC formation after a bolus injection of 10 mg/kg ERY to rats.
Troleandomycin, a macrolide antibiotic and classic inhibitor of CYP3A, is also an inducer of CYP3A2 and CYP3A4 (Watkins et al., 1986). Whether ERY also acts as a CYP3A inducer remains controversial. Although several studies reported an increase in total P450s after ERY treatment (Danan et al., 1981; Amacher et al., 1991), no change in total P450s was observed in other studies (Yamano et al., 2000; Takedomi et al., 2001). It is not clear why these discrepancy exists across different studies. Differences in the dosing regimens and experimental design may play a role. In this study, there was a small but insignificant increase in total P450s and CYP3A2 mRNA after chronic ERY (Table 3). The lack of significant change in CYP3A2 mRNA rules out induction by transcriptional activation, but it remains theoretically possible that substrate binding results in induction by protein stabilization, as noted for CYP2E1 (Chien et al., 1997). Although formation of an MIC appears to stabilize CYP3A in vivo and results in an increase in immunoquantifiable protein, there is no increase in activity because the accumulating complex is inactive (Larrey et al., 1983). There is no evidence to support induction of active CYP3A by mechanisms other than transcriptional activation, and therefore in our model the effects of ERY are restricted to reversible and irreversible inhibition.

Our 4-day dosing regimen resulted in the in vivo formation of MIC that was easily quantified spectrophotometrically (Fig. 4A). After discontinuation of ERY dosing, the amount of MIC present in the liver declined in a first-order manner with an estimated elimination rate constant of 0.035 h⁻¹ (Fig. 4B) assuming a zero order rate for the Liver declined in a first-order manner with an estimated elimination rate constant of 0.035 h⁻¹ (Fig. 4B) assuming a zero order rate for the CYP3A synthesis that is unaffected by ERY administration and a first-order rate of enzyme degradation. Our estimate is within the range of CYP3A elimination rate constants in rats (0.0348–0.072 h⁻¹) estimated in previous studies (Shiraki and Guengerich, 1984; Correia, 1991). An accurate estimation of the degradation rate constant is critical for the prediction of the extent and time course of irreversible inhibition in vivo (Mayhew et al., 2000; Zhang et al., 2009; Zhang et al., 2009). As demonstrated by the sensitivity analysis, a 10-fold variation in k_{deg} resulted in a MDZ AUC ratio change from 8.3 to 18.8. However, this parameter is generally not obtained, and this result contributes to the poor performance and uncertainty of previous PBPK predictive models of irreversible inhibition in vivo.

To test whether the observed decrease in the in vivo CL_{int} of MDZ can be predicted by using in vitro-estimated inactivation parameters, k_{inact} and k_{el} were estimated in RLMs from livers of rats in the control group. A physiologically based approach, taking into account the temporal disposition of the inhibitor and the change in the active enzyme pool in the liver, was applied. Conventionally, simple algebraic calculations at a single-inhibitor concentration (usually C_{max}) have been commonly adopted, ignoring the changing concentrations of the inhibitor after its administration (Thummel and Wilkinson, 1998; Mayhew et al., 2000; Bjornsson et al., 2003; Wang et al., 2004; Obach et al., 2005). This approach leads to false prediction in the inhibition potential. To test whether a simplistic equation with one inhibitor concentration is sufficient to predict the interaction between ERY and MDZ, a well defined equation for the prediction of the extent of MBI (eq. 10), adapted from a study by Wang et al. (2004), was used. In the application of eq. 10, the parameter values were equivalent to those used in the PBPK model, except for a constant single-inhibitor concentration, calculated from the time-averaged ERY concentration during the time interval when MDZ was administered. As shown in Table 4, using a time-averaged ERY concentration significantly overestimates the magnitude of the decrease in MDZ CL_{int} (15.4-fold versus observed 2.3-fold). In contrast, the PBPK model applied in the current study predicted a 2.6-fold decrease in CYP3A activity after repeated ERY treatment, which is in excellent agreement with the observed in vivo CL_{int} ratio (2.3-fold; Table 2) and the fold decrease in the in vitro-estimated CYP3A activity (2.1-fold; Fig. 4A). These results suggest both the feasibility and meaningfulness of the physiological prediction strategy applied for the prediction of MBI and confirmed the importance of incorporating the temporal disposition of inhibitor into the prediction.

To summarize, single- and multiple-dose treatments of ERY resulted in significant reduction in the CL_{int} of MDZ in rats through reversible and irreversible inhibition of CYP3A, respectively. CYP3A inactivation by ERY is mediated through MIC formation without significant changes in the CYP3A2 mRNA. In addition, the physiologically stable, chronically cannulated rat model proved to be a valuable tool for the study of the changes in in vivo enzyme kinetics. Finally, the PBPK model using in vitro-estimated CYP3A inhibition parameters successfully predicted the decrease in MDZ CL_{int} after chronic administration of ERY. Overall, the current work has demonstrated the utility and feasibility of a physiological approach for the prediction of in vivo DDIs involving MBI by using in vitro-estimated enzyme inhibition parameters. This in vitro-in vivo extrapolation approach may be applicable to the prediction of possible clinical DDIs in humans.

**Appendix**

Equations used for the estimation of the pharmacokinetic parameters of MDZ:

\[
V_C = \frac{Dose}{A + B} \tag{12}
\]

\[
V_{ss} = V_C \times \left( 1 + \frac{k_{12}}{k_{11}} \right) \tag{13}
\]

\[
V_{PER} = V_{ss} - V_C \tag{14}
\]

\[
k_{12} = \frac{A \times \beta + B \times \alpha}{A + B} \tag{15}
\]

\[
k_{12} = \alpha + \beta - k_{12} - k_{el} \tag{16}
\]

\[
k_{el} = \frac{\alpha \times \beta}{k_{12}} \tag{17}
\]

where V_{ss} is the volume distribution at steady state and k_{el} is the elimination rate constant from central compartment.

**References**


midazolam and macrolides based on in vitro studies using human liver microsomes. Drug Metab Dispos 31:945–954.


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