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ORIGINAL ARTICLE

Integration of *In Vitro* Binding Mechanism Into the Semiphysiologically Based Pharmacokinetic Interaction Model Between Ketoconazole and Midazolam

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***In vitro* screening for drug–drug interactions is an integral component of drug development, with larger emphasis now placed on the use of *in vitro* parameters to predict clinical inhibition. However, large variability exists in K_i reported for ketoconazole with midazolam, a model inhibitor–substrate pair for CYP3A. We reviewed the literature and extracted K_i for ketoconazole as measured by the inhibition of hydroxymidazolam formation in human liver microsomes. The superset of data collected was analyzed for the impact of microsomal binding, using Langmuir and phase equilibrium binding models, and fitted to various inhibition models: competitive, noncompetitive, and mixed. A mixed inhibition model with binding corrected by an independent binding model was best able to fit the data (K_{ic} = 19.2 nmol/l and K_{in} = 39.8 nmol/l) and to predict clinical effect of ketoconazole on midazolam area under the concentration–time curve. The variability of reported K_i may partially be explained by microsomal binding and choice of inhibition model.**

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The use of *in vitro* and *in vivo* extrapolation methods in early drug discovery has expanded in an effort to provide information on drug clearance and potential drug–drug interactions earlier in development. The US Food and Drug Administration's Guidance on Drug–Drug Interactions[1] recommends the use of *in vitro* studies as a primary means to identify cytochrome P450 (CYP) inhibitors and substrates. *In vitro* studies evaluating the interaction between an inhibitor (*I*) and substrate (*S*) of an enzyme are commonly conducted early in drug development. The inhibition parameters identified in these studies can be extrapolated using semimechanistic or physiologically based pharmacokinetic models to estimate the clinical effect of drug–drug interaction. However, these predictions are limited by the reliability of the *in vitro* metabolic parameters.[2] For example, despite ketoconazole's well-established inhibition of human CYP3A isoforms, quantitative relationships for inhibition potency are not well established even for a single substrate, e.g., midazolam (**Table 2**).

Drug metabolism usually follows Michaelis–Menten kinetics such that the rate of metabolism (v) can be defined as:

$$v = \frac{V_{max} S}{K_m + S} \quad (1)$$

where V_{max} is the maximum rate of metabolism and K_m is the substrate concentration at which the reaction rate is at half of V_{max}. Reversible inhibitors may be classified as competitive or noncompetitive inhibitors. Competitive inhibitors directly inhibit substrate binding to the enzyme active site, leading to a reduction in the apparent K_m:

$$v = \frac{V_{max} S}{K_m \left(1 + \frac{I}{K_{ic}}\right) + S} \quad (2)$$

where K_{ic} is the dissociation rate constant for inhibitor binding to the substrate site. Noncompetitive inhibitors allow substrate binding but prevent product formation, effectively reducing V_{max}:

$$v = \frac{V_{max} S}{\left(1 + \frac{I}{K_{in}}\right)(K_m + S)} \quad (3)$$

where K_{in} represents the dissociation constant for inhibitor binding to the enzyme–substrate complex. The more general mixed model of inhibition (*M*) assumes simultaneous competitive and noncompetitive mechanisms:

$$v = \frac{V_{max} S}{K_m \left(1 + \frac{I}{K_{ic}}\right) + S\left(1 + \frac{I}{K_{in}}\right)} \quad (4)$$

Historically, nominal concentrations of substrate and inhibitor added to microsomal incubations have been used to determine *in vitro* kinetic rate constants. Only more recently

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has the importance of microsomal protein binding been recognized. However, fraction unbound is still not routinely determined, and many studies continue to use total rather than unbound drug concentrations when determining kinetic parameters. Correction of kinetic parameters for nonspecific binding has been based on either point-binding estimates at single microsomal protein concentrations and a nonsaturable phase equilibrium model[3,4] or a Langmuir type model that assumes saturable binding.[5,6] In this case, independent binding (BI) of substrate (*S*) and inhibitor (*I*) are expressed by the following equations with a scale factor (*P*) representing the inactive microsomal protein concentration used as a surrogate for the quantity of microsomal binding sites.

$$S_b = \frac{P\bar{B}^S_{max} S_f}{K_{d,S} + S_f} \quad (5)$$

Table 1 Estimated binding in human liver microsomes

	Midazolam	Ketoconazole
Phase equilibrium model	$S_f = \frac{S_b}{1 + K_{p,S} \times P}$	$I_f = \frac{I_b}{1 + K_{p,I} \times P}$
K_p (estimate ± SE)	0.789 ± 0.068	0.802 ± 0.155
t Value (Pr > *t*)	11.59 (0.00003)	5.19 (0.002)
Residual error	0.1728	7.7
Akaike Information Criterion	−1.88	51.4
Langmuir model	$S_b = \frac{P\bar{B}^S_{max} S_f}{K_{d,S} + S_f}$	$I_b = \frac{P\bar{B}^I_{max} I_f}{K_{d,I} + I_f}$
$\bar{B}_{max}$ (estimate ± SE)	42.32 ± 177.3	50.60 ± 9.47
t Value (Pr > *t*)	0.239 (0.821)	5.34 (0.003)
K_d (estimate ± SE)	49.43 ± 224.1	12.83 ± 8.98
t Value (Pr > *t*)	0.221 (0.834)	1.43 (0.212)
Residual error	0.1876	4.254
Akaike Information Criterion	0.083	43.8

$$I_b = \frac{P\bar{B}^I_{max} I_f}{K_{d,I} + I_f} \quad (6)$$

where subscripts f and b refer to free and bound concentrations, K_d represents the dissociation constant for nonspecific binding to microsomes, and $\bar{B}_{max}$ represents the maximum bound concentration of substrate or inhibitor per unit mass of inactive microsomal protein. If the metabolite structure is similar to the substrate, it is reasonable to use the same binding curve (Eq. 4) to also correct for metabolite binding (BIM).

At high concentrations, substrate and inhibitor may compete for binding sites. Under this competitive binding (BC) assumption, binding can be represented by Eqs. 7 and 8, derived in a manner analogous to competitive enzyme inhibition.[7]

$$S_b = \frac{P\bar{B}^S_{max} S_f}{K_{d,S}\left(1 + \frac{I_f}{K_{d,I}}\right) + S_f} \quad (7)$$

$$I_b = \frac{\bar{B}^I_{max} I_f}{K_{d,I}\left(1 + \frac{S_f}{K_{d,S}}\right) + I_f} \quad (8)$$

The variability of the inhibition parameter, K_i, for ketoconazole and various substrates has been recently reviewed.[8] The author attributed 20% of the variation to either incubation duration or microsomal concentration. The remaining variation was hypothesized to be due to such factors as enzyme expression levels, inhibition model assumptions, or specific differences due to the substrate. Other authors have suggested that microsomal preparation and preservation procedures as well as incubation buffer composition may also contribute as significant factors. To mitigate this variability, it has been suggested to use standardized incubation conditions with either low microsomal protein concentrations or correction for microsomal binding.[9] However, quantitative

Table 2 Reported *in vitro* kinetic parameters for 1′-hydroxymidazolam formation and inhibition by ketoconazole in human liver microsomes

Study	Model	V_{max} (nmol/mg/min)	K_m (μmol/l)	K_i (nmol/l)	Data extracted	Data set identification/notes
Gascon and Dayer (1991)	Noncompetitive	0.43–0.5	4.2–6.1	100	No	Data unclear
Wrighton and Ring (1994)	Noncompetitive	NR	10	110	Yes	F
von Moltke *et al.* (1996)	Competitive + substrate inhibition	2.81	3.3	3.7	Yes	B
Prueksaritanont *et al.* (1997)	Noncompetitive	NR	NR	100	Yes	E
Gibbs *et al.* (1999)	Noncompetitive	NR	NR	14.9	No	No data
Wang *et al.* (1999)	Noncompetitive	0.696	7.46	180	No	No data
Perloff *et al.* (2000)	Competitive + substrate inhibition	2.43	6.35	5.4	Yes	D
Li *et al.* (2004)	Noncompetitive	0.636	4.1	5.2	No	No data
Galetin *et al.* (2005)	Noncompetitive	NR	NR	59	Yes	A
Ogasawara *et al.* (2007)	Noncompetitive	NR	NR	32	Yes	C

NR, not reported.

examples that apply these recommendations to reduce variability in K_i are lacking.

The midazolam/ketoconazole system is an important model to study due to the combination of the strength and selectivity of ketoconazole as an inhibitor of CYP3A and the selectivity of midazolam metabolism by CYP3A. The current study examines available data on the *in vitro* inhibition of 1′-hydroxymidazolam formation by ketoconazole and the impact of microsomal binding and model selection on parameter estimation. Available data are extracted from publications and re-evaluated following correction for microsomal binding. Coupled relationships between nonspecific binding and competitive, noncompetitive, or mixed inhibition models are used to evaluate the superset of available data. The resulting inhibition parameters deduced from the superset are compared with the results in the individual studies. We also examine the sensitivity of a previously published mechanistic–dynamic pharmacokinetic model to the three drug–drug interaction models and estimated parameters.

RESULTS

Nonspecific binding

A literature search identified three studies reporting experimental values for nonspecific binding for ketoconazole and four studies for midazolam. All studies used dialysis methods to determine the fraction unbound. Data for each drug were fitted to a Langmuir-type model (Eqs. 4 and 5; **Figure 1**) and a phase equilibrium model.[4] The midazolam data fit quite well to a phase equilibrium model, with no advantage being gained from the Langmuir-type model as evidenced by the higher value for the Akaike Information Criterion (AIC) and the nearly identical residual sum of squares (**Table 1**). By contrast, the more general Langmuir model data better fit the ketoconazole data. The residual sum of squares was approximately half of the value for the phase equilibrium model and the AIC value was considerably lower.

In general, the results indicate that a phase equilibrium model may not always be the best description for drug binding to human liver microsomes. The predicted K_p values from the phase equilibrium model for midazolam and ketoconazole were similar; however, the confidence limits differed for the two drugs. In the case of ketoconazole, the phase equilibrium model was not able to accommodate the strong dependence of the f_u curve on the concentration of the drug. Although the data better fit a phase equilibrium model for midazolam, the concentration range for the data set was narrow, supporting only a limited applicability.

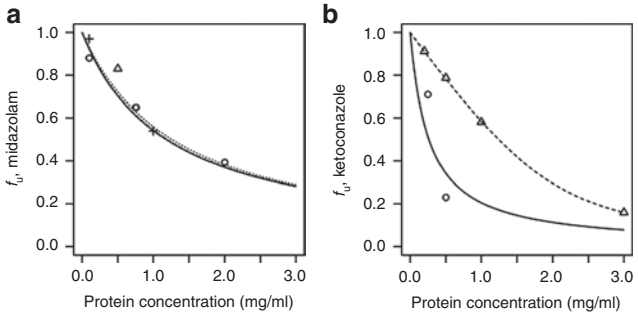

Figure 1 Nonspecific binding of (**a**) midazolam and (**b**) ketoconazole. Fraction unbound, f_u, is the ratio of the free concentration, S_f, to the total concentration, S_t. Circles, triangles, and plus marks represent concentrations of 1, 2, and 7.5 µmol/l of midazolam. Circles and triangles represent observed data at 1 and 100 µmol/l of ketoconazole. Lines indicate predicted binding at the different concentrations of midazolam (solid line: 1 µmol/l; dashed line: 2 µmol/l; dotted line: 7.5 µmol/l) or ketoconazole (solid line: 1 µmol/l; dashed line: 100 µmol/l).

By contrast, the B_{max} estimates for the two drugs were similar in magnitude as would be expected for adsorption of similarly sized molecules. Likewise, a significant difference was predicted in the K_d estimates for the drugs consistent with the expectation of higher binding affinity for ketoconazole. Given this consistency and the limitations in both data sets, the Langmuir model was chosen for further use in the analysis with the caution that further expansion of the data sets would be needed to establish tight constraints on the parameter estimates.

In vitro metabolism

Ten reports of *in vitro* ketoconazole–midazolam inhibition studies were identified in the literature (**Table 2** and **Supplementary Table S1** online). Of these, six (A–F) contained graphical data that could be extracted for reanalysis.[10–15] The available data were combined and analyzed by combinations of various binding and inhibition models: no binding (BN); independent binding (BI); independent binding corrected for metabolite binding (BIM); competitive binding (BC); competitive inhibition (C); noncompetitive inhibition (NC); and mixed inhibition (M). Summary results of the combined fit of the data extracted from studies A–F to the alternative inhibition and binding models are listed in **Table 3**. The mixed inhibition model consistently resulted in the overall best fit with the lowest AIC value regardless of the assumed binding model. The next best model was the noncompetitive model which showed little differentiation from the mixed model. Incorporation of any of the binding models significantly improved the fit in comparison with the case with no binding assumption; however, the independent binding assumption resulted in the lowest overall AIC value. The absolute best fit resulted from a mixed inhibition model and an independent binding model (M-BIM) for ketoconazole, midazolam, and its metabolite.

The data for studies A–F along with the predictions based on the M-BIM model are shown in **Figure 2**. Data are normalized by the parameter, α, which adjusts the rate, v, in each study according to the observed individual study V_{max} compared to the overall $\bar{V}_{max}$. The mean (0.81), median (0.87), and standard deviation (0.39) of the α parameters in this model indicate a skewed, wide distribution with a range of (0.20–1.22). The normalization improved the fit significantly by reducing the RSS from 8.0 to 1.52 after the first iteration. A further 6.5% reduction in the RSS was accomplished with up to 35 iterations.

Visually, the fits appear to be comparable with the original study fits using a common set of kinetic parameters for all the data sets. This is remarkable given the range of incubation conditions used in the studies and the wide variation in reported K_i values and inhibition models. No systematic structure of the underlying residuals was observed when plotted vs. midazolam concentration, ketoconazole concentration, study designator, or microsomal protein concentration.

Table 3 Parameter estimates for microsomal binding and inhibition models

Binding model	BN			BI			BC			BIM		
Inhibition model	**C**	**NC**	**M**	**C**	**NC**	**M**	**C**	**NC**	**M**	**C**	**NC**	**M**
V_{max} (nmol/mg/min)					2.31 ± 0.11						2.79 ± 0.15	
K_m (μmol/l) (±8%)	4.31	3.85	4.27	3.23	3.32	3.45	3.47	3.12	3.51	3.30	3.35	3.48
K_{ic} (nmol/l) (±8%)	10.8	–	33.1	4.1	–	18.9	5.4	–	14.2	4.2	–	19.2
K_{in} (nmol/l) (±6%)	–	91.7	135.4	–	33.2	39.6	–	43.8	71.6	–	33.5	39.8
RSS	3.14	1.87	1.71	3.27	1.49	1.44	2.68	1.97	1.60	3.25	1.47	1.42
Akaike Information Criterion	125	71.2	64.0	130	47.9	46.5	109	77.0	57.0	129	46.2	45.1

BC, competitive binding; BI, independent binding; BIM, metabolite binding; BN, no binding; C, competitive inhibition; M, mixed inhibition; NC, noncompetitive inhibition.

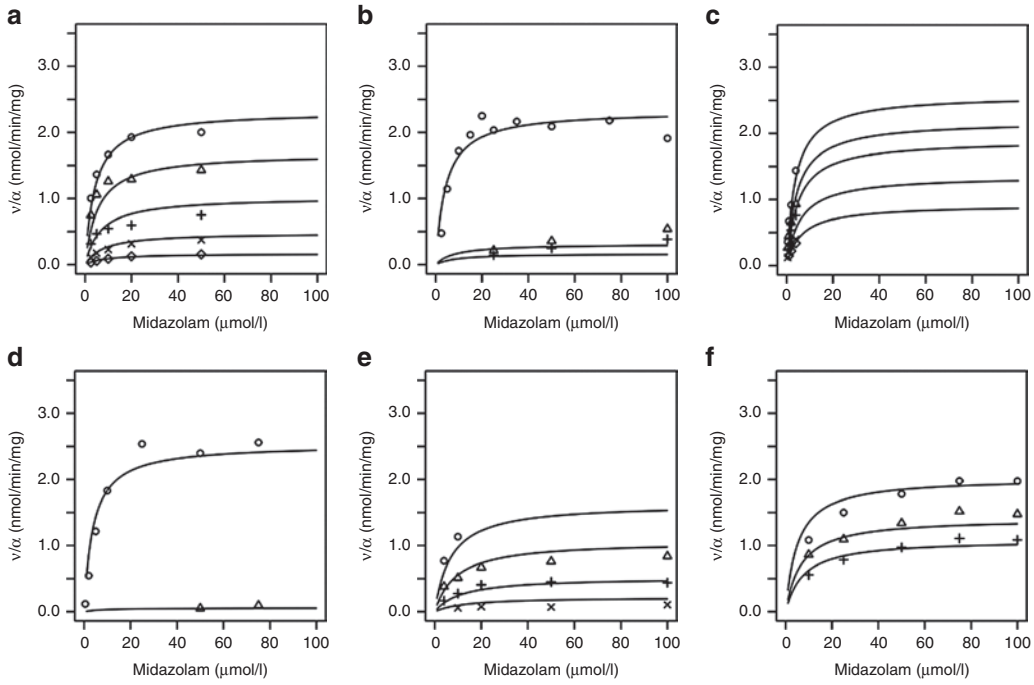

Figure 2 Model (M-BIM) fits of the data from studies A-F (**a**–**f**, respectively) for the inhibition of α-hydroxymidazolam formation by ketoconazole in human liver microsomes. Data are normalized by the parameter, α, which adjusts the rate, v, in each study according to the ratio of the observed study V_{max} and the average V_{max} for all studies. Midazolam concentration is the total concentration, whereas the rate is based on the measured (free) concentration of the metabolite. Each curve represents a different total concentration level of inhibitor as reported in each study.

The model fit and residuals analyses give support for the interpretation that the separate experimental conditions were similar enough to support the combined analysis.

Studies A–D, conducted at low microsomal protein concentration, show consistent maximum measured rates between the studies. This reflects the relative insensitivity of the metabolite binding to microsomal protein concentration at low concentrations. Studies E and F, conducted at higher protein concentration, show the effect of metabolite binding at high microsomal protein concentrations. In these studies, a greater fraction of the metabolite is bound so the observed (free) rate decreases at higher substrate concentrations. This characteristic is the principal feature distinguishing model M-BIM from the other competing models M-BI and NC-BI which predict equivalent maximum observed (free) rates, independent of microsomal protein concentration (data not shown).

There are a few notable data points from the individual studies that appear to be inconsistent with the model fit, notably the points corresponding to high midazolam/inhibitor concentrations and the data point at 100 μmol/l midazolam and no inhibitor in study B. The latter point was explained in the original study as an effect related to substrate-induced inhibition at higher midazolam concentrations. No other study observed this effect at a lower substrate concentration, supporting truncation of the data sets at 100 μmol/l midazolam. The former data points no doubt contributed to the interpretation of a competitive inhibition model in study B; however, other than these few data points, other outliers may be explained by experimental error in the original studies.

In vitro and *in vivo* extrapolation of drug–drug interactions

The sensitivity of a previously published semimechanistic metabolism model to the different inhibition models was

evaluated. K_{ic} and K_{in} from the independent BIM results were incorporated into midazolam clearance (Eqs. 8–10). The model was used to simulate a study in which 200 mg of ketoconazole was administered daily for 7 days before the administration of intravenous midazolam.[16] All midazolam concentrations were normalized to a dose of 1 mg. Predicted midazolam concentrations in the absence of the inhibitor were similar to those observed in the trial (**Figure 3a**). The predicted area under the concentration–time curve (AUC) of midazolam in the absence of inhibitor was 0.031 ng/ml/h, which was comparable with the observed value of 0.032 ng/ml/h.

The mixed inhibition model most accurately predicted the change in the AUC of IV midazolam following 200-mg ketoconazole daily for 6 days (**Figure 3b**). The predicted AUC ratios (AUCRs) using the competitive and noncompetitive models were outside of the 80–120% acceptance criteria (**Table 4**). Using the mixed inhibition model, the predicted AUCR was 13% lower than the observed AUCR, well within the 80–120% acceptance criteria.

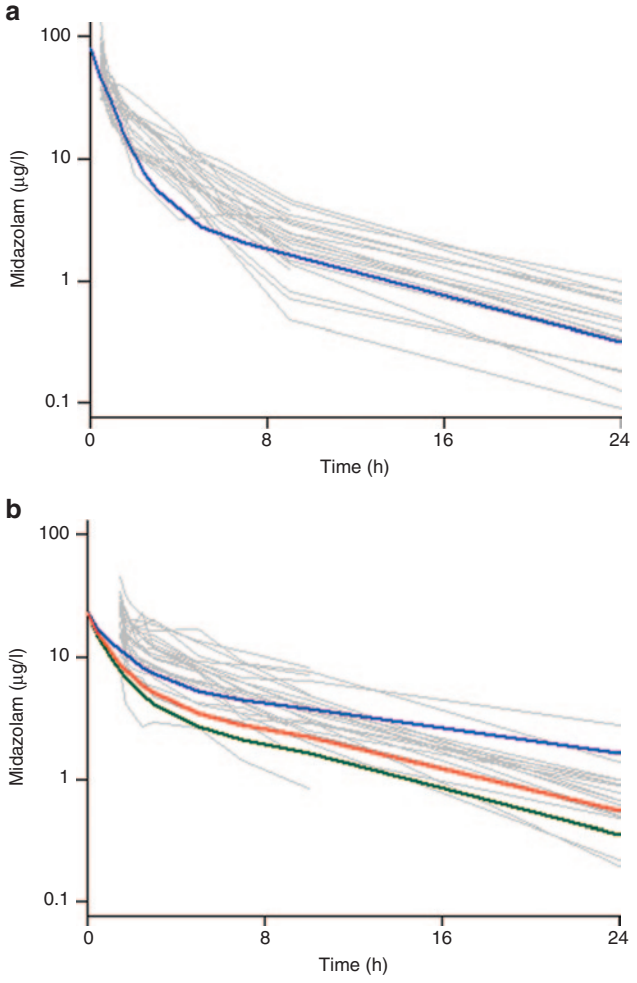

Figure 3 Predicted and observed concentration vs. time plots of (**a**) i.v. midazolam alone and (**b**) in the presence of 200 mg ketoconazole. (**a**) Gray lines indicate observed concentrations of midazolam, blue line represents model prediction for midazolam alone. (**b**) Gray lines indicate observed concentrations of midazolam, blue line indicates predicted concentrations based on the competitive inhibition model, red line indicates predicted concentrations based on the mixed inhibition model, and green line indicates predicted concentrations based on the noncompetitive inhibition model.

DISCUSSION

Midazolam and ketoconazole are the prototypical CYP3A substrate and inhibitor used for *in vitro* drug interaction studies of new drug compounds. *In vitro* studies examining the inhibition potency of ketoconazole on midazolam report approximately a 50-fold range in K_i (**Table 2**). In addition, the mechanism of inhibition is described as competitive in some reports, whereas others find it to be noncompetitive. To quantitatively predict the clinical effect of inhibition, it is important to identify accurate *in vitro* inhibition parameters. We thus conducted a literature review and reanalysis of available data to identify the optimal *in vitro* inhibition model and parameters for ketoconazole. This reanalysis incorporated normalization of data for binding and fitting of competitive, noncompetitive and mixed inhibition model functions.

The normalization procedure decreased the error in the fitting procedure due to the variation in CYP3A expression, interpreted as the variation of apparent V_{max} between studies. The value of V_{max} reported here for the M-BIM model is consistent with two of the originally reported estimates (studies B and D). These studies were conducted at relatively low microsomal protein concentration and based on the M-BIM model showed similar expression levels as compared with the average with α values of 0.88 and 0.87, respectively. Both of these conditions favor the generation of similar predictions by the M-BIM model to the original studies. Although some studies reporting dissimilar V_{max} values[17–19] were not included in the analysis, other representative studies with varying apparent V_{max} values were included, namely studies C (ref. 15) and E (ref. 12) with α values of 0.21 and 0.55, respectively. Of note, study E used a much higher microsomal protein concentration (1 mg/ml) than used in the other studies or, in the case of study C, only evaluated a small range of inhibitor concentrations. Exclusion of this individual study from the data set did not alter the overall results.

The K_m range of the original studies varied from 3.3 to 10 µmol/l (**Table 2**). On the basis of data sets extracted from six reports, we estimated K_m using various binding models. The K_m values estimated by these models were similar (range: 3.12–3.51 µmol/l). Of note, when not corrected for binding, the K_m estimates were higher (3.85–4.31 µmol/l). However, these values are still lower than several reported K_m estimates for midazolam (**Table 2**). Although some of the shift can be explained in terms of the incorporation of the substrate and inhibitor binding models for those studies using higher microsomal protein concentrations,[20] the

Table 4 AUCRs of i.v. midazolam following 200-mg ketoconazole

	AUCR	Bias
Competitive model	4.5	45%
Noncompetitive model	2.0	–35%
Mixed model	2.5	–20%
Observed data	3.1 (2.8–3.3)	–

AUCR, area under the concentration–time curve ratio.

reasons for the remaining variation in the original studies is unclear. It has been proposed that part of the variation could be due to varying ratios of expression levels of CYP3A4 and CYP3A5 isoforms.[21] However, this explanation does not explain reported values for $K_m > 6$.[10,13,15] Given the good fit of the M-BIM model and the consistency of K_m across the other models considered in this study, it is likely that previous reported higher values of K_m were artifacts of limited individual data sets and the least squares fitting of the hyperbolic Michaelis–Menten equations.[7] The present estimate for K_m of 3.48 μmol/l benefits from the wide ranging data sets, incorporation of drug binding, varying microsomal protein concentrations, and incubation times directly in the M-BIM model.

With regard to the K_i values, much of the variability in the original studies also appears to be explained in terms of the present models. For example, the K_{ic} values from the competitive inhibition models which incorporated binding were consistent. When not corrected for binding, the K_{ic} was much higher and inconsistent with previously reported values. Likewise, the K_{in} values from the noncompetitive inhibition models which incorporated binding were consistent and did not vary to the great extent reported in the original studies. Although our analysis identified the mixed inhibition model to best fit the data, the noncompetitive model fit similarly well. Of note, these models determined similar K_{in}.

The variability of model choice and the reported range of inhibition constants in the original reports are likely due to the limited data sets, uncertainties in least squares fitting, and the different experimental conditions (**Supplementary Table S1** online).

The *in vitro* parameter estimates identified using the BIM model were applied to the *in vivo* pharmacokinetic interaction model proposed by Chien *et al.*[22] CYP3A inhibition was modeled using competitive, noncompetitive, and mixed inhibition models, and predictions were compared with the data observed in a healthy volunteer study of i.v. midazolam following a 200-mg daily dosage regimen of ketoconazole. The mixed inhibition model predicted a 1.8- and 2.3-fold greater inhibition of midazolam clearance as compared with the competitive or noncompetitive models, respectively, indicating that choice of inhibition model can influence drug interaction predictions.

CONCLUSION

Determination of Michaelis–Menten parameters for drug substrate and inhibitor pairs is subject to challenges related to the size of data sets, control of experimental conditions, assumed mechanisms for drug, substrate, and metabolite binding to liver microsomes, assumed drug inhibition mechanisms, and the least squares fitting procedure. Much of the variation between studies may be explained by incorporating additional factors such as relative expression levels, microsomal protein concentration, and incubation time into coupled binding and inhibition models. For the midazolam/ketoconazole system, a mixed inhibition model coupled with independent binding of the drug, inhibitor, and metabolite is sufficient to explain the variation of reported V_{max}, K_m, and inhibitor constants across a range of reported studies. The appropriate choice of inhibition model and parameters is important for *in vitro* and *in vivo* extrapolation models predicting clinical drug interactions.

Care should be taken when relying on parameters derived from small studies or studies that do not account for microsomal binding of drug substrate, inhibitor, and metabolite binding. Studies conducted at low microsomal protein concentration are likely reliable in describing the data; however, incorporation of a range in concentration improves the robustness of the least-squares fitting procedures and may provide for improved accuracy in the underlying parameters.

METHODS

Model description. Literature reports have classified the *in vitro* inhibition mechanism of ketoconazole as competitive (C) and noncompetitive (NC) or mixed (Eqs. 2–4).

To estimate free substrate and inhibitor concentrations, the binding curves (Eqs. 5–8) were solved and the free concentrations determined by subtracting out the bound portion. For a given data set, P is usually held constant so the number of enzyme-binding sites is dependent only on the corresponding expression level of the samples in a given study. Differences in expression levels between studies have not normally been considered. The inhibitor disassociation constants, K_{ic} or K_{in}, are usually of the most interest, and protein expression levels are normally considered to be constant within a single study. To correct for potential differences in expression levels between studies, V_{max}, in Eqs. 2–4 is replaced by $\alpha \overline{V}_{max}$, where $\overline{V}_{max}$ represents the overall average V_{max} across studies and α is a study-dependent scaling parameter. The study α parameter can be interpreted as the observed relative expression level of active enzyme as compared with the average study. Other kinetic parameters are assumed to be appropriately corrected by adjustment for binding Eqs. 5 and 6 and equal across studies.

Literature search. Studies reporting *in vitro* K_i values for the inhibition of midazolam 1′-hydroxylation by ketoconazole determined in human liver microsomes were identified through the Metabolism and Transport Drug Interaction Database (http://www.druginteractioninfo.org), PubMed, and reference lists of relevant papers. For each K_i, the type of inhibition and other information on incubation conditions, including substrate (midazolam) and inhibitor (ketoconazole) concentrations, buffer composition, microsomal protein concentrations, incubation time, and nicotinamide adenine dinucleotide phosphate source were recorded and are shown in **Supplementary Table S1** online. **Table 2** provides a summary of the reported parameters, inhibition model, and notes on the studies which were excluded from the analysis. One study was excluded due to uncertainty in extraction of data from figures and potentially nonrepresentative microsomal protein concentration.[17] Other excluded studies were a result of the absence of raw data in the original report.[18,19,21] Studies reporting nonspecific binding of midazolam[9,23–26]or ketoconazole[3,27] in human liver microsomal systems were identified either through PubMed or relevant literature references.

Data extraction. Representative studies reporting discrete data points either in the form of a table or graph for *in vitro* metabolism or nonspecific binding were included in the analysis. Data in table format were extracted visually, whereas data in graphs were extracted electronically utilizing the software *g3data*[28] with reproducibility in general better than 2%. The precision and reproducibility of the data extractions were comparable with a prior software validation.[29] In cases where data were originally plotted as reciprocals, error was greater near the axes. Data points with a precision >6% were omitted due to this limitation in the presentation of the original data.

Binding analysis. Extracted binding data for both midazolam and ketoconazole were fit to Eqs. 4–7 utilizing the statistics software R version 2.12 (ref. 30) and the nonlinear least squares function to determine the binding parameters for either an BI or BC model. The goodness of fit and confidence in the parameter estimates were interpreted based on the output of the nonlinear least squares function and visual inspection of the fitted curves and residuals. R code and raw data are included in the **Supplementary Data** online.

In vitro *metabolism analysis*. Three cases of binding were considered according to the BI, BC, or BIM models. In the first two cases, free concentrations of midazolam and ketoconazole were determined from the reported total concentrations according to the binding parameters derived from either binding model BI or BC. In the third case, total metabolite formation rates for each study were determined from the reported (free) metabolite formation rates, v, where applicable, by the use of the midazolam-independent binding curve, BIM, with scaling to concentration units via the study microsomal protein concentration and incubation time.

Initial estimates of the kinetic parameters for the superset of data were determined assuming equal V_{max} values between studies for each inhibition model corresponding to Eqs. 1–3, using R and the nonlinear least squares library. The initial estimate for V_{max} was taken as the overall $\overline{V}_{max}$ in subsequent recursions. The initial parameter set was applied and constrained in a secondary fit for each study subset to obtain an initial α estimate for each study. In subsequent recursions, the y ordinates, corresponding to v, were linearly transformed as v/α to scale for different study V_{max} values, and the whole superset of data were again fitted to Eqs. 1–3. Recursion was repeated until convergence was achieved. Residuals were calculated in the normal manner relative to the unscaled rate, v, and the total midazolam/ketoconazole concentration, S and I. Comparison of the AIC were used to compare model fits corresponding to a matrix of combinations of inhibition and binding models (C-BI, C-BIM, C-BC, NC-BI, NC-BIM, NC-BC, M-BI, M-BIM, and M-BC) as well as to the fits assuming no binding (C-BN, NC-BN, and M-BN).

Note in this analysis scheme that only $\overline{V}_{max}$ is constrained to the initial fit. The K_m, K_{ic}, K_{in}, and α parameters reflect the overall fits with linear scaling of the rates to account for differences in average expression levels between studies. R code and raw data are available in **Supplementary Data** online.

In vitro *and* in vivo *extrapolation.* The sensitivity of *in vivo* drug–drug interaction prediction to inhibition model was examined using a mechanistic dynamic interaction model. The mechanistic dynamic model for competitive inhibition has been previously published.[22] In the case of a competitive inhibitor, midazolam intrinsic clearance (CL_{int}) by CYP3A is estimated by Eq. 9:

$$CL_{int} = \frac{f_m \times V_{max} \times C_{MDZ} \times f_{u,MDZ}}{f_{u,MDZ} \times K_m \times \left(1 + \frac{f_{u,KTZ} \times C_{KTZ}}{k_{ic}}\right) + f_{u,MDZ} \times C_{MDZ}} \quad (9)$$

where f_m is the fraction metabolized by CYP3A; C_{MDZ} and C_{KTZ} indicate the concentration of midazolam and ketoconazole, respectively, in the liver; $f_{u,MDZ}$ and $f_{u,KTZ}$ are the fraction unbound of midazolam and ketoconazole which were assumed to be 0.04 and 0.01, respectively.[22]

Assuming ketoconazole acts as a noncompetitive inhibitor would alter CL_{int} such that:

$$CL_{int} = \frac{f_m \times V_{max} \times C_{MDZ} \times f_{u,MDZ}}{\left(f_{u,MDZ} \times K_m \times \left(1 + \frac{f_{u,KTZ} \times C_{KTZ}}{k_{in}}\right) + f_{u,MDZ} \times C_{MDZ} \times \left(1 + \frac{f_{u,KTZ} \times C_{KTZ}}{k_{in}}\right)\right)} \quad (10)$$

A mixed inhibitor would affect the CL_{int} such that

$$CL_{int} = \frac{f_m \times V_{max} \times C_{MDZ} \times f_{u,MDZ}}{\left(f_{u,MDZ} \times K_m \times \left(1 + \frac{f_{u,KTZ} \times C_{KTZ}}{k_{ic}}\right) + f_{u,MDZ} \times C_{MDZ} \times \left(1 + \frac{f_{u,KTZ} \times C_{KTZ}}{k_{in}}\right)\right)} \quad (11)$$

All other equations and parameters were consistent with Chien *et al.*'s report. A single subject was simulated for each inhibition type using the mean parameters and the K_{in}, K_{ic}, and K_m calculated using the BIM binding model. The models were written in R (see **Supplementary Data** online). The extent of clinical interaction is estimated by the ratio of the AUC (AUCR) of the substrate (midazolam) in the presence of inhibitor (ketoconazole) to its AUC in the absence of inhibitor. The predicted AUCR was compared with the observed AUCR obtained in a clinical study of midazolam and ketoconazole.[16] Acceptance criteria were based on the predicted AUCR falling within 80–120% of the observed AUCR. The model was also evaluated by visual inspection of concentration vs. time plots.

Author contributions. S.K.Q. and S.K. wrote the manuscript. L.L., S.D.H., S.K.Q., and S.K. designed the research. S.K.Q., S.K., and C.C. performed the research. S.K.Q., S.K., and C.C. analyzed the data.

Conflict of interest. The authors declare no conflict of interest.

Study Highlights

WHAT IS THE CURRENT KNOWLEDGE ON THE TOPIC?

✓ A nearly 50-fold range exists in the reported K_i of ketoconazole for midazolam in human liver microsomes. Study conditions such as incubation time and protein concentration play a role in this variability.

WHAT QUESTION THIS STUDY ADDRESSED?

✓ We aimed to identify an optimal K_i for ketoconazole. Published data were corrected for protein binding of midazolam and ketoconazole and fitted to competitive, noncompetitive, and mixed inhibition models.

WHAT THIS STUDY ADDS TO OUR KNOWLEDGE

✓ After correcting for binding, a mixed inhibition model best fit the data.

HOW THIS MIGHT CHANGE CLINICAL PHARMACOLOGY AND THERAPEUTICS

✓ This study demonstrates the importance of correcting for protein binding in *in vitro* studies and demonstrates the utility of a phase equilibrium model for binding correction. We also demonstrate that the *in vitro–in vivo* extrapolation of a mixed inhibition model.

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Supplementary information accompanies this paper on the *CPT: Pharmacometrics & Systems Pharmacology* website (http://www.nature.com/psp)