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Research Paper

Mechanism of cytochrome P450-3A inhibition by ketoconazole

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Abstract

Objectives Ketoconazole is extensively used as an index inhibitor of cytochrome P450-3A (CYP3A) activity *in vitro* and *in vivo*, but the mechanism of ketoconazole inhibition of CYP3A still is not clearly established.

Methods Inhibition of metabolite formation by ketoconazole (seven concentrations from 0.01 to 1.0 μM) was studied in human liver microsomes ($n = 4$) at six to seven substrate concentrations for triazolam, midazolam, and testosterone, and at two substrate concentrations for nifedipine.

Key findings Analysis of multiple data points per liver sample based on a mixed competitive–noncompetitive model yielded mean inhibition constant K_i values in the range of 0.011 to 0.045 μM. Ketoconazole IC50 increased at higher substrate concentrations, thereby excluding pure noncompetitive inhibition. For triazolam, testosterone, and midazolam α-hydroxylation, mean values of α (indicating the 'mix' of competitive and noncompetitive inhibition) ranged from 2.1 to 6.3. However, inhibition of midazolam 4-hydroxylation was consistent with a competitive process. Determination of K_i and α based on the relation between 50% inhibitory concentration values and substrate concentration yielded similar values. Pre-incubation of ketoconazole with microsomes before addition of substrate did not enhance inhibition, whereas inhibition by troleandomycin was significantly enhanced by pre-incubation.

Conclusions Ketoconazole inhibition of triazolam α- and 4-hydroxylation, midazolam α-hydroxylation, testosterone 6β-hydroxylation, and nifedipine oxidation appeared to be a mixed competitive–noncompetitive process, with the noncompetitive component being dominant but not exclusive. Quantitative estimates of K_i were in the low nanomolar range for all four substrates.

Keywords competitive inhibition; cytochrome P450-3A; ketoconazole; mechanism-based inhibition; noncompetitive inhibition

Introduction

Ketoconazole is extensively used as an 'index' inhibitor of human cytochrome P450-3A (CYP3A) isoforms in the academic, regulatory, and drug development communities.[1–3] Although ketoconazole has been used in this context for some twenty years, the kinetic mechanism of CYP3A inhibition by ketoconazole in human liver microsomal preparations still is not clearly established. Published in-vitro studies of ketoconazole inhibition of CYP3A have yielded differing and conflicting conclusions as to whether inhibition can be explained by competitive, noncompetitive, or mixed competitive–noncompetitive mechanisms.[4] Accurate assignment of the mechanism of inhibition affects understanding of the fundamental nature of the substrate–enzyme and inhibitor–enzyme interactions, as well as the interpretation of in-vitro inhibition constants (K_i), the validity of which is dependent on the correctness of the underlying model of inhibition.[5]

We have evaluated the mechanism of CYP3A inhibition by ketoconazole in human liver microsomal preparations. To our knowledge this is among the few studies to assess this question using CYP3A substrates from different categories, with a sufficient number of

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substrate and inhibitor concentrations such that enzyme kinetic analysis could proceed without a predetermined decision as to whether the inhibition mechanism was competitive, noncompetitive, or mixed.[6]

Materials and Methods

Preparation of human liver microsomes

Liver samples from four individual human donors with no known liver disease were provided by the International Institute for the Advancement of Medicine (Exton, PA, USA); the Liver Tissue Procurement and Distribution System, University of Minnesota (Minneapolis, MN, USA); or the National Disease Research Interchange (Philadelphia, PA, USA). The ages of the four donors were: 21, 34, 37, and 62 years. All were male and the cause of death was either physical trauma or sudden illness.

Microsomes were prepared by ultracentrifugation; microsomal pellets were suspended in 0.1 M potassium phosphate buffer containing 20% glycerol and were stored at −80°C until use.[7]

Preparation of reagents

Chemical reagents used for microsomal incubations and HPLC analysis were purchased from commercial sources. Triazolam, midazolam, testosterone, nifedipine, ketoconazole, and troleandomycin (triacetyloleandomycin) were purchased from commercial sources or kindly provided by their pharmaceutical manufacturers.

Stock solutions of the drug entities were prepared in methanol, and subsequently diluted with methanol as needed. Solutions were stored at −18°C.

In-vitro study design

Four CYP3A substrates were selected for study: triazolam, midazolam, testosterone and nifedipine.[8,9] The choice of substrates was based on categories proposed by Kenworthy *et al*.[6] For studies of triazolam α-hydroxylation and 4-hydroxylation, midazolam α-hydroxylation and 4-hydroxylation, and testosterone 6β-hydroxylation, incubations were performed at multiple substrate concentrations. At each concentration, additional incubations were done with co-addition of ketoconazole at concentrations of 0, 0.01, 0.025, 0.05, 0.1, 0.25, 0.5 or 1.0 μM. Within this range ketoconazole is established as a relatively specific CYP3A inhibitor. Studies were done with microsomal preparations from four different human liver samples and all individual incubations were performed in duplicate.

Nifedipine dehydrogenation studies were done at two fixed concentrations of 25 and 250 μM. Incubations were performed with co-addition of varying concentrations of ketoconazole as described above.

For testosterone as substrate, an additional study was conducted to evaluate the effect of 20 min pre-incubation of microsomes with ketoconazole, prior to the addition of testosterone (100 μM) on the inhibitory effect of ketoconazole at concentrations of 0.05 and 0.25 μM. Troleandomycin was used as the positive control in this study. Troleandomycin concentrations were 0, 1, 5, 10, 25, and 50 μM.

Incubation procedures

Incubation mixtures contained 50 mM phosphate buffer, 5 mM $MgCl_2$, 0.5 mM nicotinamide adenine dinucleotide phosphate, and an isocitrate and isocitric dehydrogenase regenerating system.[7,8] Substrates (Table 1) and ketoconazole or troleandomycin, as appropriate, were added to a series of incubation tubes. The solvent was evaporated to dryness at 40°C under mild vacuum conditions.

After addition of the incubation mixture components to yield a final volume of 250 μl, reactions were initiated by the addition of microsomal protein (0.1–0.25 mg/ml). The incubation duration ranged from 5 to 40 min, depending on the intrinsic clearance of the substrate.[8,10–12] All reactions were shown to be in the linear range with respect to protein concentration and incubation duration.

At the appropriate time reactions were stopped by cooling on ice and the addition of 100 μl acetonitrile. Internal standard was added, the incubation mixture was centrifuged, and the supernatant was transferred to an autosampling vial for HPLC analysis of metabolites (Table 1). The analytic column was stainless steel, 15 cm × 3.9 mm, containing reverse-phase C18 Novapak (Waters Associates, Milford, MA, USA) (Table 1).

Analysis of data

For triazolam, midazolam, and testosterone, analyses were performed for each individual liver sample using combinations of reaction velocity (V) at each substrate concentration ([S]) and inhibitor concentration ([I]). The following equation, consistent with mixed competitive–noncompetitive inhibition, was fitted to the data points by nonlinear regression using SAS PROC NLIN:[13–15]

Table 1 Summary of reaction characteristics and chromatographic conditions

Substrate	Product(s)	Internal standard	Mobile phase composition	Ultraviolet absorbance wavelength
Triazolam	α-OH-triazolam, 4-OH-triazolam	Phenacetin	70% buffer[a] (10 mM), 20% acetonitrile, 10% methanol	220 nm
Midazolam	α-OH-midazolam, 4-OH-midazolam	Phenacetin	45% buffer[a] (10 mM), 20% acetonitrile, 35% methanol	220 nm
Testosterone	6β-OH-testosterone	Androstenedione	55% water, 45% acetonitrile	240 nm
Nifedipine	Oxidized nifedipine	Diazepam	45% water, 55% methanol	270 nm

[a]Potassium dihydrogen phosphate (pH unadjusted).

$$V = \frac{V_{max} \cdot [S]}{[S]\left(1 + \frac{[I]}{\alpha \cdot K_i}\right) + K_m\left(1 + \frac{[I]}{K_i}\right)} \quad (1)$$

Iterated variables were: V_{max}, the maximum reaction velocity; K_m, the Michaels–Menten constant; K_i, the inhibition constant; and α, a number $\geq$ 1.0 indicating the 'mix' of competitive and noncompetitive mechanisms. In the case of testosterone, a sigmoidal (Hill) kinetic pattern was observed without and with the addition of ketoconazole. For purposes of nonlinear regression analysis of testosterone data, equation 1 was empirically modified by addition of an exponent to [S] and to S50 (replacing K_m).

For determination of 50% inhibitory concentration (IC50) values, reaction velocities with co-addition of inhibitor were expressed as a percentage ratio (R_v) of the control velocity with no inhibitor present. The relationship of R_v to inhibitor concentration ([I]) was analysed by nonlinear regression to determine the IC50 value, based on the following equation:

$$R_v = 100\left(1 - \frac{E_{max}[I]^b}{[I]^b + IC^b}\right) \quad (2)$$

Iterated variables were: E_{max}, the maximum degree of inhibition; IC, the inhibitor concentration producing an R_v value of 50% of $(100 - E_{max})$; and b, an exponent. The actual IC50 was calculated as:

$$IC50 = IC \Big/ \left(2E_{max} - 1\right)^{1/b} \quad (3)$$

IC50 values were determined based on mean data points across four liver samples at corresponding inhibitor concentrations.

The relationship between IC50 and substrate concentration can be used as an alternative method for determining α and K_i for mixed competitive–noncompetitive inhibition.[16] That relationship is:

$$IC50 = \frac{K_i\left(1 + \frac{[S]}{K_m}\right)}{\left(1 + \frac{[S]}{\alpha \cdot K_m}\right)} \quad (4)$$

This is illustrated in Figure 1. Note that for pure competitive inhibition (α = 'infinity') this reduces to:

$$IC50 = K_i\left(1 + \frac{[S]}{K_m}\right) \quad (5)$$

whereas for pure noncompetitive inhibition (α = 1.0), this reduces to:

$$IC50 = K_i \quad (6)$$

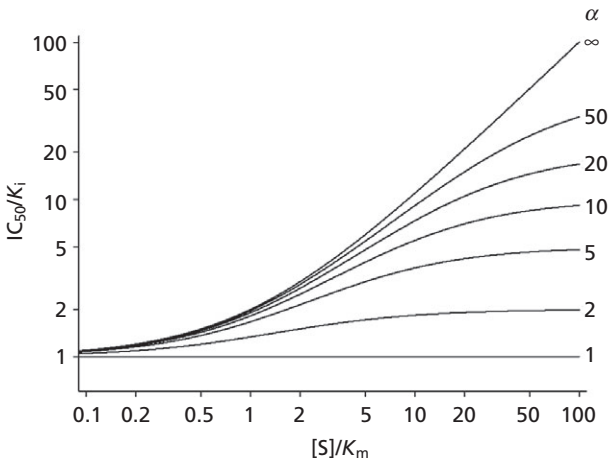

Figure 1 For a mixed competitive–noncompetitive inhibitor, the relationship between the [S]/K_m ratio (x-axis) and the IC50/K_i ratio, at varying values of α ranging from 1.0 to 'infinity'. The functions were predicted based on equation 4.

For triazolam, midazolam and testosterone, ketoconazole IC50 values were available at multiple substrate concentrations. Using mean K_m (or S50) values determined as described above (eqn 1) as fixed entries in equation 4, this equation was fitted to data points ([S] and IC50) by nonlinear regression. Iterated variables were α and K_i. For nifedipine, the same procedure was applied using the available data points, with the K_m value from a previously reported study.[8]

Results

Nonlinear regression based on equation 1 (modified by an exponent in the case of testosterone) yielded convergent solutions (Figures 2 and 3).

For triazolam, K_m values for the 4-OH-triazolam pathway exceeded K_m values for the α-OH-triazolam pathway (Table 2). This was similar to previous studies from our laboratory and elsewhere. [8,10,11,17] Ketoconazole inhibition of triazolam metabolite formation was consistent with the mixed competitive–noncompetitive scheme (Figure 2). The mean values of α were 2.11 and 2.14 for the α-hydroxylation and 4-hydroxylation pathways, respectively, indicating a predominance of noncompetitive inhibition in the balance.

Consistent with previous reports, K_m values for the midazolam α-hydroxylation pathway were on average more than tenfold lower than for the 4-hydroxylation pathway (Table 2, Figure 3).[8,11,12,18,19] Ketoconazole inhibition of α-OH-midazolam formation was explained by the mixed model, with a mean α of 6.30. However, inhibition of midazolam 4-hydroxylation was consistent with a competitive process, inasmuch as estimates of α from nonlinear regression were very large.

As described previously, testosterone 6β-hydroxylation was best described by a sigmoidal (Hill) kinetic pattern.[5,8,20–25] Inhibition by ketoconazole was consistent with the mixed competitive–noncompetitive model (Figure 2).

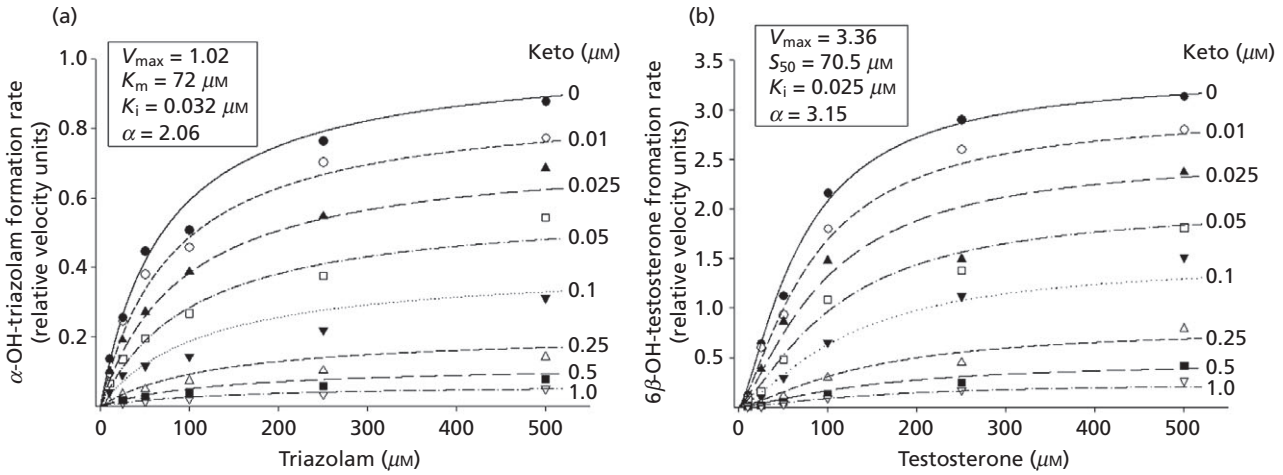

Figure 2 Rates of formation of α-OH-triazolam from triazolam (a) and formation of 6β-OH-testosterone from testosterone (b) in a human liver sample. Lines represent the functions determined by nonlinear regression based on equation 1. For testosterone, equation 1 was empirically modified by addition of an exponent to account for sigmoidal kinetics.

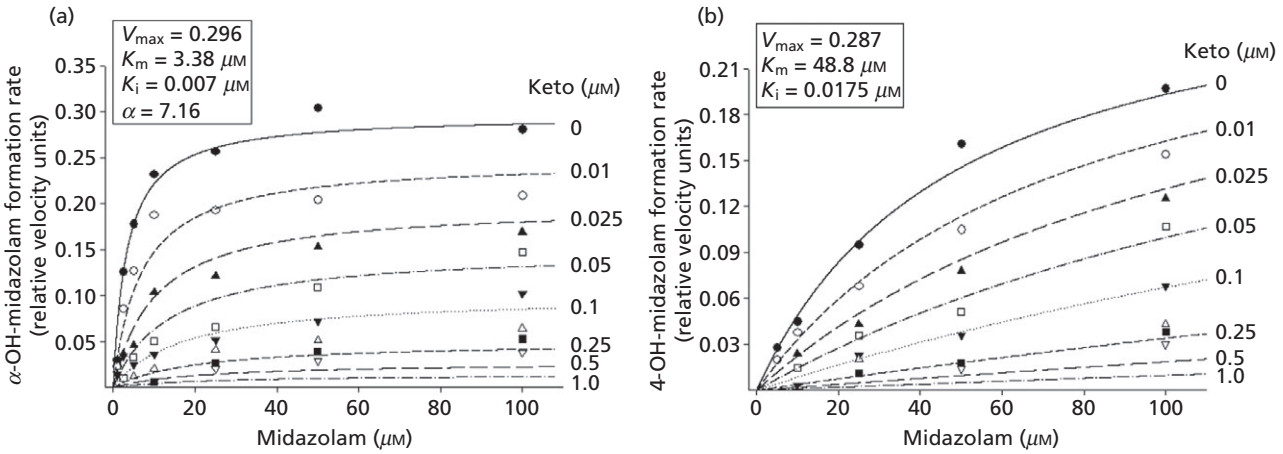

Figure 3 Rates of formation of α-OH-midazolam (a) and 4-OH-midazolam (b) from midazolam in a human liver sample. Lines represent the functions determined by nonlinear regression based on equation 1.

Table 2 Enzyme kinetic parameters for in-vitro biotransformation of cytochrome P450-3A substrates with co-addition of ketoconazole as inhibitor

Substrate	Product	Based on equation 1 (mean ± SE, n = 4)			Based on equation 4	
		K_m (μM)	K_i (μM)	α	K_i	α
Triazolam	α-OH-triazolam	63.1 (± 3.8)	0.031 (± 0.003)	2.14 (± 0.13)	0.041	1.51
	4-OH-triazolam	319 (± 45)	0.045 (± 0.005)	2.11 (± 0.43)	0.052	1.41
Midazolam	α-OH-midazolam	3.34 (± 0.15)	0.011 (± 0.004)	6.30 (± 1.77)	0.0083	8.54
	4-OH-midazolam	51.3 (± 8.2)	0.019 (± 0.003)	[b]	0.019	[b]
Testosterone	6β-OH-testosterone	69 (± 7)[a]	0.019 (± 0.002)	3.07 (± 0.23)	0.028	2.11
Nifedipine	Oxidized nifedipine	–	–	–	0.072	4.25

[a]S50. [b]Values of α for 4-OH-midazolam formation were very large, consistent with competitive inhibition.

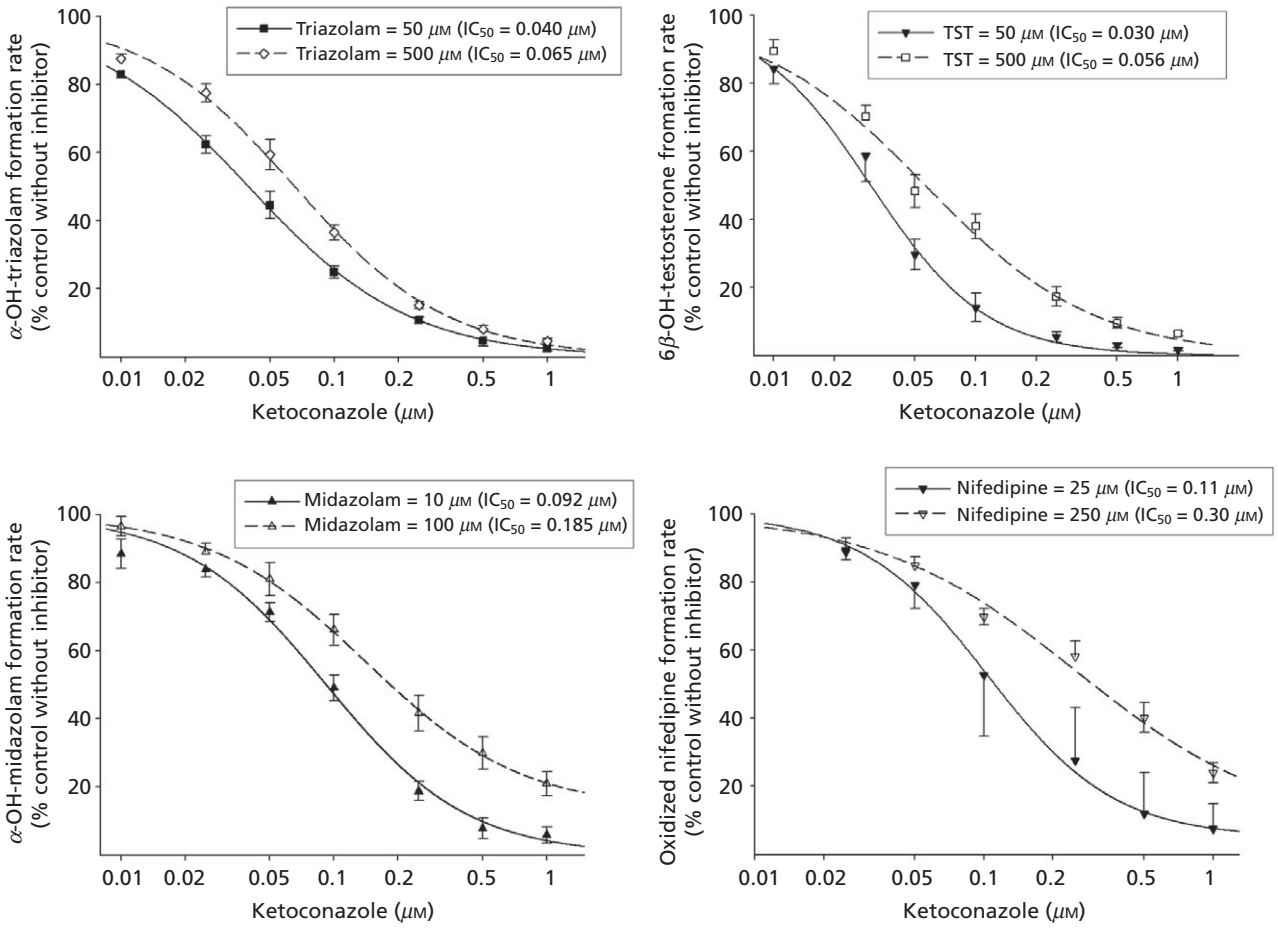

Figure 4 Inhibition curves for ketoconazole vs four different substrates at low and high concentrations. Points are mean (± SE, $n = 4$) percentage ratios of reaction velocity with coaddition of ketoconazole divided by the control velocity without ketoconazole. Lines are the functions consistent with equation 2 determined by nonlinear regression. TST, testosterone.

Values of α fell between 2.7 and 3.7 (mean 3.07), indicating the predominance of noncompetitive inhibition in the mixed model (Table 2).

For all four CYP3A substrates, ketoconazole IC50 values increased at higher concentrations of substrate (Figure 4). Analysis of the relation between IC50 and [S] based on equation 4 yielded estimates of K_i and α that were similar to those determined from the analysis using equation 1 (Table 2). For 4-OH-midazolam formation, equation 4 again yielded very large estimates of α, indicating competitive inhibition. Ketoconazole inhibition of nifedipine oxidation was explained via equation 4 as a mixed competitive–noncompetitive process, with $K_i = 0.072$ μM and $\alpha = 4.25$.

Inhibition of testosterone 6β-hydroxylation by troleandomycin was clearly enhanced by pre-incubation (Figure 5), consistent with mechanism-based inhibition.[26–30] However, inhibition by ketoconazole was not enhanced by pre-incubation (Figure 5), confirming reversible rather than mechanism-based inactivation of CYP3A by ketoconazole.[30–33]

Discussion

While ketoconazole is generally recognized as a strong inhibitor of human CYP3A isoforms, numerous studies published over the last two decades have indicated extensive variability in quantitative K_i values for ketoconazole vs different CYP3A substrates, as well as differences among studies of the same substrate.[1,2,4] Ketoconazole K_i may also differ even for two parallel metabolic pathways for the same substrate such as alprazolam, triazolam, midazolam and terfenadine.[10–12,34,35]

Quantitative determination of K_i values from in-vitro studies is dependent on the assumed or established underlying mechanism of inhibition. Any given set of data points will yield a different estimate of K_i if the mechanism of inhibition is assumed to be competitive or noncompetitive. Therefore it is probable that some component of the variability among studies in K_i values is explained by differing assignments of inhibitory mechanism.

Among 51 published studies reporting in-vitro K_i values for ketoconazole inhibition of CYP3A substrate

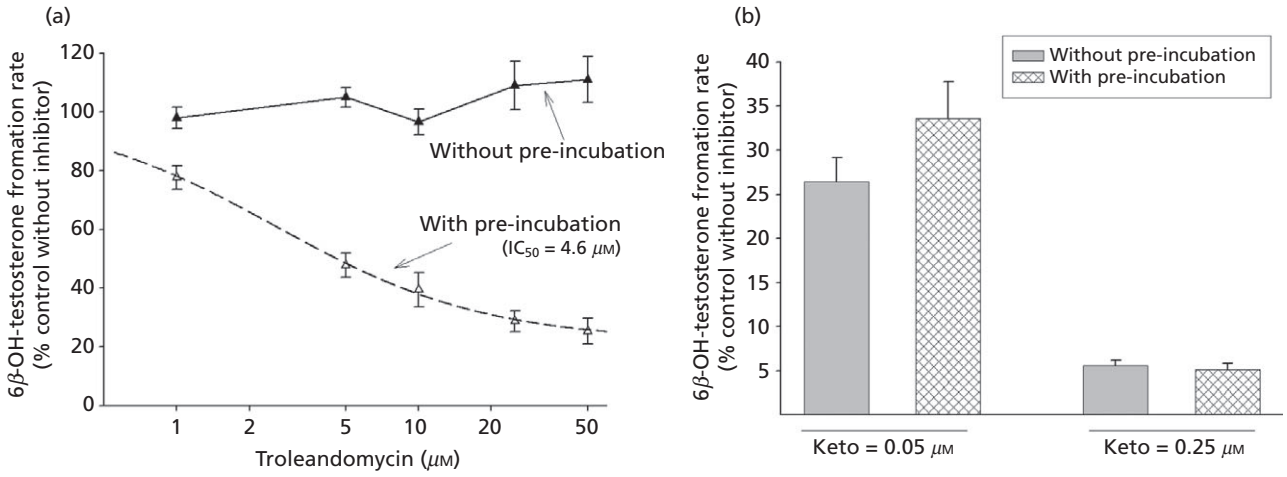

Figure 5 (a) Effect of troleandomycin on the rate of 6β-OH-testosterone formation from testosterone. (b) Mean percentage ratios of reaction velocities with coaddition of ketoconazole divided by the control velocity without ketoconazole. For (a): points are mean ($\pm$ SE, $n = 4$) percentage ratios of reaction velocities with coaddition of troleandomycin divided by the control velocity without troleandomycin. Studies were done without and with pre-incubation of troleandomycin with microsomes before addition of the substrate. Dashed line is the function consistent with equation 2 determined by nonlinear regression. For (b): mean $\pm$ SE, $n = 4$. Ketoconazole was used at 0.05 or 0.25 μM. Studies were done without and with pre-incubation of microsomes with ketoconazole.

biotransformation, the majority assigned a fully competitive mechanism.[4] In some of these cases, the competitive process was assumed or 'forced' *a priori*, without an evaluation of whether that mechanism was most appropriate for the data. In other studies, the choice of competitive, noncompetitive or mixed inhibition was based on the appearance of data when plotted after some form of linearizing transformation (typically a Dixon plot of the reciprocal of reaction velocity vs inhibitor concentration at different substrate concentrations). These transformations may provide useful information (including estimates of K_i), but may be misleading or distortive since they involve calculations of reciprocals. Data points having the smallest numerical values – and therefore subject to greater measurement inaccuracy – become inappropriately magnified in importance. Small experimental errors in such points are thereby expanded, and may bias slopes and intercepts of reciprocal plots.

This study evaluated the kinetics of ketoconazole inhibition of human CYP3A *in vitro*, using four different substrates to assess the inhibitory mechanism. The pre-incubation study with testosterone as substrate verified that inhibition by ketoconazole was reversible as opposed to mechanism-based, as reported previously from our own laboratory and elsewhere.[30–33] The finding of increased IC50 values at higher concentrations of all four substrates (Figure 4) effectively excluded purely noncompetitive inhibition as the underlying mechanism, since the IC50 value would be unchanged (and equal to K_i) regardless of substrate concentration (eqn 6). For triazolam, midazolam, and testosterone, nonlinear regression of all data points allowed simultaneous determination of K_i and of the 'mix' of competitive and noncompetitive processes, with no *a priori* assumptions about the mechanism, and without the potential biases of reciprocal transformations. The outcome of the analysis demonstrated mixed-mechanism inhibition for triazolam, testosterone and midazolam α-hydroxylation. The mean values of α were in the range of 2–7, and the K_i values for ketoconazole were in the low nanomolar range. It is of interest that the K_i values vs the 4-OH-triazolam pathway were higher than for the α-OH-triazolam pathway. This confirmed our previous observations and emphasized that K_i values for a given inhibitor of CYP3A may differ even between two parallel metabolic pathways for the same substrate.[10,11] Finally, we observed that ketoconazole inhibition of midazolam 4-hydroxylation, as opposed to α-hydroxylation, was consistent with a competitive process. These phenomena might be explained by cooperativity and multisite kinetic behaviour of human CYP3A.[5,20,23,25,36]

The relation between the IC50 value and substrate concentration provided an alternative approach to determination of the K_i and α-values. Since the IC50 value increased at higher substrate concentrations, pure noncompetitive inhibition was excluded. For triazolam α-hydroxylation and 4-hydroxylation, testosterone 6β-hydroxylation and midazolam α-hydroxylation, application of equation 4 indicated mixed-mechanism inhibition, with a predominance of the noncompetitive component. The numerical values of K_i and α were similar to those determined from equation 1. Application of equation 4 to midazolam 4-hydroxylation indicated competitive inhibition, as was found using equation 1. Therefore, two independent approaches to data analysis yielded similar or identical conclusions regarding the mechanism of inhibition by ketoconazole, as well as the numerical values of K_i.

The four CYP3A substrates used in this study were based on the hypothetical categories originally proposed by Kenworthy *et al*.[6] The conclusion from that study was that probe substrates could be divided into two major groups, one of which included triazolam and midazolam, and another which included testosterone. Nifedipine was described as not falling into either of the discrete groups. Those authors made the point that there was a high degree of consistency among CYP3A substrates in terms of extensive inhibition caused by established strong CYP3A inhibitors, and minimal inhibition

caused by weak inhibitors. We have reported a very high degree of correlation of IC50 values for 22 different CYP3A inhibitors using midazolam and testosterone as CYP3A substrates (see Figure 2.2 of Volak *et al*.[15]) based on data published by Obach *et al*.[37] A similar point has been made in other publications.[20,38,39] In this study, ketoconazole was demonstrated to be a strong inhibitor of CYP3A – with K_i or IC50 values in the low nanomolar range – regardless of the hypothetical category of the substrate. Although current Food and Drug Administration guidelines recommend the use of more than one CYP3A substrate for in-vitro studies of CYP3A inhibition, our findings suggested that a single CYP3A index substrate may be sufficient for in-vitro characterization of a potential CYP3A inhibitor.[40] There appeared to be low probability of acquiring unique information from study of one or more additional CYP3A substrates.

Conclusions

Although ketoconazole is a strong CYP3A inhibitor regardless of the specific substrate, there are nonetheless variations among substrates in the quantitative potency of ketoconazole inhibition even under standardized in-vitro study conditions. The mechanism of inhibition, though reversible, in general cannot be characterized as fully competitive or fully noncompetitive. This study has suggested that a mixed competitive–noncompetitive mechanism was likely to be applicable. The noncompetitive component appeared dominant in most instances, but the quantitative 'mix' of competitive and noncompetitive processes will vary among substrates and even between parallel biotransformation pathways for the same substrate.

Declarations

Conflict of interest

The Author(s) declare(s) that they have no conflicts of interest to disclose.

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