ROLE OF ITRACONAZOLE METABOLITES IN CYP3A4 INHIBITION


Department of Pharmaceutics (N.I., K.E.T.) and Department of Medicinal Chemistry (K.L.K., K.E.A., W.L.N.), University of Washington, Seattle US.
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*Corresponding Author
University of Washington
Department of Pharmaceutics
H272 Health Sciences Building, Box 357610
Seattle, WA 98195-7610
Ph: (206) 543-9434
Fax: (206) 543-3204
thummel@u.washington.edu

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Nonstandard abbreviations used:
ITZ, Itraconazole; OH-ITZ, hydroxy-itraconazole, keto-ITZ, keto-itraconazole ND-ITZ, N-desalkyl-itraconazole, CYP, cytochrome P450, \(K_m\), Michaelis constant; \(K_i\), inhibition constant, \(Cl_{int}\), intrinsic clearance
ABSTRACT

Itraconazole (ITZ) is a potent inhibitor of CYP3A in vivo. However, unbound plasma concentrations of ITZ are much lower than its reported in vitro Ki, and no clinically significant interactions would be expected based on a reversible mechanism of inhibition. The purpose of this study was to evaluate the reasons for the in vitro-in vivo discrepancy. The metabolism of ITZ by CYP3A4 was studied. Three metabolites were detected: hydroxy-itraconazole (OH-ITZ), a known in vivo metabolite of ITZ, and two new metabolites: keto-itraconazole (keto-ITZ) and N-desalkyl-itraconazole (ND-ITZ). OH-ITZ and keto-ITZ were also substrates of CYP3A4. Using a substrate depletion kinetic approach for parameter determination, ITZ exhibited an unbound Km of 3.9 nM and Clint of 69.3 ml•min⁻¹•nmol CYP3A4⁻¹. The respective unbound Km values for OH-ITZ and keto-ITZ were 27 nM and 1.4 nM and the Clint values were 19.8 and 62.5 ml•min⁻¹•nmol CYP3A4⁻¹. Inhibition of CYP3A4 by ITZ, OH-ITZ, keto-ITZ and ND-ITZ was evaluated using hydroxylation of midazolam as a probe reaction. Both ITZ and OH-ITZ were competitive inhibitors of CYP3A4, with unbound Ki (1.3 nM for ITZ and 14.4 nM for OH-ITZ) close to their respective Km. ITZ, OH-ITZ, keto-ITZ and ND-ITZ exhibited unbound IC₅₀ values of 6.1 nM, 4.6 nM, 7.0 nM and 0.4 nM, respectively, when coincubated with human liver microsomes and midazolam (substrate concentration <Km). These findings demonstrate that ITZ metabolites are as potent or more potent CYP3A4 inhibitors than ITZ itself, and thus may contribute to the inhibition of CYP3A4 observed in vivo after ITZ dosing.
INTRODUCTION

Itraconazole (ITZ, Figure 1) is a widely used triazole antifungal agent. It has a broad spectrum of activity and is effective against many fluconazole-resistant strains of fungi (Haria et al., 1996). In humans, the pharmacokinetic behavior of ITZ is characterized by an intermediate bioavailability (55%), low-intermediate clearance (23 l/h), extensive tissue distribution and a large apparent volume of distribution (11 l/kg) (Heykants et al., 1989; Poirier and Cheymol, 1998). Itraconazole is mainly eliminated from the body via metabolism and more than 30 different metabolites have been proposed (Heykants et al., 1989; Poirier and Cheymol, 1998). Only one of these metabolites has been studied in man; hydroxy-itraconazole (OH-ITZ, Figure 1). Hydroxy-itraconazole possesses antifungal activity similar to that of ITZ and it circulates in plasma at higher concentrations than ITZ (Heykants et al., 1989; Poirier and Cheymol, 1998). At present, it is not known which P450 enzymes contribute to OH-ITZ formation in vivo or in vitro.

The steady-state concentrations of ITZ cannot be predicted from single-dose data (Poirier and Cheymol, 1998) and multiple-dose administration results in a 26-60% increase in elimination half-life and a 69-80% reduction in oral clearance of ITZ (Hardin et al., 1988). In addition, ITZ exhibits disproportional increases in AUC with increasing single oral doses (Heykants et al., 1989; Haria et al., 1996) and dose-dependent bioavailability (Schäfer-Körting, 1993). These findings suggest that metabolism of ITZ may be saturable at clinically relevant plasma concentrations or that one of the metabolites of ITZ inhibits its metabolism. Clinical evidence suggests that ITZ is mainly eliminated by CYP3A. Phenytoin, a known inducer of CYP3A4, caused a 17-fold increase in ITZ oral clearance (Ducharme et al., 1995). Administration of other CYP3A4 inducers, such as rifampicin (Tucker et al., 1992), carbamazepine and phenobarbital (Bonay et al., 1993) result in undetectable ITZ plasma concentrations, probably due to increased first-pass extraction as well as faster systemic elimination.
The evidence for involvement of CYP3A in ITZ elimination in vivo together with the non-linear kinetics of ITZ are consistent with well-established inhibition interactions between ITZ and other CYP3A substrates. Drugs whose pharmacokinetics is significantly affected by ITZ include cyclosporine (Kwan et al., 1987; Trenk et al., 1987; Florea et al., 2003), tacrolimus (Banerjee et al., 2001; Mahnke et al., 2003), midazolam (Olkkola et al., 1996; Backman et al., 1998), buspirone (Kivistö et al., 1999) methylprednisolone (Lebrun-Vignes et al., 2001), lovastatin (Neuvonen and Jalava, 1996) and simvastatin (Neuvonen et al., 1998). The observed increase in the in vivo AUC of these drugs is between 2- and 30-fold after concomitant administration of ITZ.

ITZ is a relatively potent inhibitor of CYP3A4 in vitro. However, Ki values determined in vitro for CYP3A4 range from 27 nM to 11 µM (Back and Tjia, 1991; von Moltke et al., 1996; Wang et al., 1999; Ishigam et al., 2001). Differences in experimental microsomal protein content (0.04-0.25mg/ml) and non-specific binding of ITZ to microsomal protein may account for this large variation in observed Ki values. Despite the relatively high affinity of ITZ for CYP3A4, its unbound plasma concentrations are generally lower (1-3 nM) than the lowest reported Ki. Assuming a reversible inhibition mechanism, a ratio of unbound ITZ concentration to Ki less than one suggests that no clinically significant interaction involving CYP3A should occur. However, this is clearly inconsistent with the observed in vivo interactions described above.

Concentrative uptake of ITZ into hepatocytes might account for the apparent in vitro-in vivo discrepancy described above. In this regard, Yamano et al. reported that, in the rat, the unbound hepatic concentration of ITZ can greatly exceed (11- to 14-fold) the unbound plasma concentration (Yamano et al., 2001). Although active uptake of ITZ into human hepatocytes may also occur, we considered an alternative hypothesis, formation of CYP3A inhibitory metabolites, that could contribute to the apparent in vitro – in vivo discrepancy.
Thus, the goals of this study were to (1) determine whether CYP3A4 catalyze the initial oxidations of ITZ and (2) evaluate whether the discrepancy between *in vitro* and *in vivo* data with respect to inhibition of CYP3A4 by ITZ is due to a failure to account for *in vivo* inhibitory ITZ metabolites, extensive nonspecific binding of ITZ to microsomal proteins *in vitro*, or rapid depletion of ITZ *in vitro*. In order to address these aims, the sequential metabolism of ITZ by CYP3A4 was studied using human liver microsomes and heterologously expressed CYP3A4 (Supersomes™). Three metabolites formed by CYP3A4 were identified. The inhibitory potencies of ITZ and its identified metabolites towards CYP3A4 were determined, and their unbound concentrations in microsomal incubations were measured in order to obtain unbound IC₅₀ values for comparison.

**MATERIALS AND METHODS**

**Chemicals**

Itraconazole and hydroxy-itraconazole were purchased from Janssen Biotech, Research Diagnostics Inc, (Flanders, NJ). Keto-itraconazole was generously provided by Dr. Jan Heeres, Janssen Pharmaceutica N. V., Beerse, Belgium. The metabolite was also prepared in our laboratory by Swern oxidation from hydroxy-itraconazole (Tidwell, 1990; Weerawarna et al., 1991). N-Desalkyl-itraconazole was prepared from an intermediate hydrazide-carboxamide, also provided by Dr. Heeres, by reaction with formamidine acetate to generate the triazolone ring, as previously reported (Heeres et al., 1984). Midazolam, 1′-hydroxymidazolam (OH-MDZ) and 1′-[²H₂]-hydroxymidazolam were gifts from Roche laboratories (Nutley, NJ). Acetonitrile was purchased from Fischer Scientific Co. (Fairlawn, NJ), ammonium acetate from J.T. Baker (Phillipsburg, NJ) and NADPH from Sigma-Aldrich (St. Louis, MO). Ultrapure water, filtered through a Barnstead Nanopure filter system was used throughout the study.
Human Liver Microsomes (HLM) and Recombinant CYPs

HLMs were prepared by a standard ultracentrifugation technique from five donor livers selected from the University of Washington Human Liver Bank (Lin et al., 2002). All were known to be devoid of significant amounts of CYP3A5 protein (CYP3A5*3/*3 genotype). The protein concentration in each HLM was measured by standard methods (Lowry et al., 1951). Equal amounts of microsomal protein from the five different preparations were pooled for subsequent experimentation. Supersomes™ containing cDNA-expressed CYP3A4 co-expressed with P450 reductase (OR) and cytochrome b<sub>5</sub> were purchased from BD Gentest Corp. (Woburn, MA). Additional Supersomes containing cDNA-expressed P450-reductases and CYPS 1A1, 1A2, 2A6 (with b<sub>5</sub>), 2B6 (with b<sub>5</sub>), 2E1 (with b<sub>5</sub>), 2C9 (with b<sub>5</sub>), 2C19 (with b<sub>5</sub>), 2D6, and 3A5 were also purchased from BD Gentest.

Identification of ITZ Metabolites Formed by CYP3A4

ITZ metabolites were identified primarily by mass spectrometry and by comparing the HPLC retention times of metabolite peaks to available reference compounds. ITZ or one of its metabolites (OH-ITZ or keto-ITZ) was incubated at 1 µM concentration with 100 pmol CYP3A4 Supersomes in 0.5 ml of 100 mM monobasic phosphate buffer (KPi), pH 7.4, with 100 mM EDTA and with or without NADPH (1 mM) for 60 min at 37°C. At the end of the incubation, the reaction was terminated by the addition of 5 ml of dichloromethane. The organic phase was separated and evaporated to dryness under a nitrogen stream and the sample residue reconstituted in 200 µl of acetonitrile. Twenty µl of the solution was injected into the Hewlett Packard series 1100 MSD system and analytes were eluted according to the method used for quantitation of metabolites (see section below). Total ion monitoring was conducted to obtain information about molecular weight (quasimolecular ions) and chloride isotope patterns together with the retention time of the metabolite.
For additional structural information, MS/MS analysis of the quasimolecular ion was conducted for ITZ and its three identified metabolites; hydroxy-itraconazole (OH-ITZ), keto-itraconazole (keto-ITZ) and N-desalkyl-itraconazole (ND-ITZ). The MS/MS spectra were recorded using a Micromass Quattro II triple quadrupole mass spectrometer operated on the positive ion electrospray atmospheric pressure ionization mode. The MS was equipped with a Shimadzu LC-10AD pump, DGU-14A degasser, SCL-10A controller, SIL-10AD autosampler and micromass Masslynx data analysis software. For the MS, the cone voltage was 30 V and the collision energy with argon 35 eV. Capillary voltage was 3.5 kV; source temperature 150°C and desolvation temperature 350°C; the drying gas was set at 400 and nebulizing gas at 25. The scan time was 2.5 seconds and inter-scan time 0.1 seconds. The fragments produced from ions with m/z 705 (ITZ), m/z 721 (OH-ITZ), m/z 719 (keto-ITZ) and m/z 649 (ND-ITZ) were recorded in separate chromatographic runs. The compounds were separated by HPLC using an Agilent Zorbax Eclipse XDB-C8 5 µm column (2.1 mm i.d. × 50 mm). The mobile phase flow was 0.2 ml/min and the following gradient was used: The initial conditions of 40% aqueous (5 mM ammonium acetate buffer, pH 6.8) and 60% acetonitrile were held for half a min after injection, after which the acetonitrile concentration was increased linearly to 70% over 1.5 min and further to 80% over 2 min. The acetonitrile concentration was then increased linearly over 1.5 min to 95% and held at 95% for 0.5 min.

Incubation Conditions for Substrate Depletion Experiments

All substrates (ITZ, OH-ITZ, and keto-ITZ) were dissolved in acetone and added to the incubations at appropriate concentrations that gave a final acetone concentration in the incubation of 1%. The incubations were performed in 100 mM KPi buffer (pH 7.4) with 1 mM EDTA. The NADPH concentration in all incubations was 1 mM. Insect cell-expressed CYP3A4 Supersomes (co-expressed with P450 reductase and cytochrome b₅) were used in all incubations.
at a concentration of 10 pmol CYP3A4/ml (0.084 mg microsomal protein/ml). Pilot incubation experiments were performed with human liver microsomes and heterologously expressed CYP3A4 Supersomes to determine steady-state conditions (protein and time linearity) and to identify potential metabolites of ITZ formed by CYP3A4. Significant and rapid substrate depletion was observed for ITZ and its metabolites. Therefore, a substrate depletion approach was adopted for determination of kinetic parameters.

For substrate depletion studies, all incubations were performed in duplicate. A starting incubation volume of 1.4 ml was used. After preincubation for 4 min at 37°C, a time 0 min sample (200 µl) was transferred to a vial containing 200 µl of ice-cold acetonitrile and the reaction was initiated with NADPH (final concentration 1 mM). At time points 1, 2, 5, 10 and 15 min, 200 µl samples were transferred to tubes containing 200 µl of ice-cold acetonitrile for quenching the reaction. The samples were vortexed, centrifuged at 12,000 g for 10 min, and the supernatant transferred to an HPLC vial for LC-MS analysis.

**Incubation Conditions for Incubation of CYP Panel with ITZ**

Formation of OH-ITZ from ITZ by CYP1A1, CYP1A2, CYP2A6, CYP2B6, CYP2C9, CYP2C19, CYP2D6, CYP2E1, CYP3A4 and CYP3A5 was tested using heterologously expressed CYP Supersomes™ obtained from BD Gentest (Woburn). Each incubation was performed in 200 µl of 100 mM KPi buffer, pH 7.4, with 50 pmol of the respective CYP isoform. The final ITZ concentration was 500 nM. The metabolic reactions were initiated with the addition of NADPH (1 mM) and allowed to proceed for 10 minutes. After 10 minutes, the reactions were quenched with the addition of 200 µl of acetonitrile and formation of OH-ITZ, keto-ITZ and ND-ITZ was measured by LC-MS. CYP3A5 catalyzed formation of OH-ITZ from ITZ (50 nM and 1 µM) with and without additional cytochrome b₅ was also examined. Additionally, depletion of ITZ by CYP3A4 and CYP3A5 (with and without supplemented b₅)
was tested at ITZ concentrations of 50 nM and 500 nM. For these experiments, ITZ was incubated with the enzyme for 30 minutes and samples were collected at 5, 15 and 30 min after initiation of metabolism by NADPH.

**Quantitation of ITZ, OH-ITZ, Keto-ITZ and ND-ITZ by Liquid Chromatography-Mass Spectrometry (LC-MS)**

ITZ, OH-ITZ, keto-ITZ, and ND-ITZ concentrations were measured by high performance liquid chromatography-mass spectrometry (LC-MS) using a Hewlett Packard series 1100 MSD system operating in the positive ion electrospray mode with selected ion monitoring and equipped with HP Chemstation data analysis software. The mobile phase consisted of acetonitrile and 5 mM ammonium acetate buffer (pH 7.0) with a gradient elution. Twenty µl of the sample were injected on column. The mobile phase flow was 0.25 ml/min and the initial mobile phase was 40% acetonitrile and 60% 5 mM ammonium acetate buffer. The acetonitrile concentration was increased linearly to 70% over 3 min and then to 85% over 1 min and finally to 95% over 1 min. After 0.5 min the acetonitrile concentration was decreased back to 40%. Analyte separation was achieved using a Zorbax Eclipse XDB-C8 5 µm column (2.1 mm i.d. × 50 mm; Agilent) equipped with a Phenomenex C8 guard column (2.1 mm i.d. × 4 mm). Ion m/z 705 was monitored for ITZ, m/z 721 for OH-ITZ, m/z 719 for keto-ITZ and m/z 649 for ND-ITZ. For the mass spectrometer, the drying gas flow was set at 10 l/min, nebulizer pressure at 25 psig, gas temperature at 300°C and the capillary voltage (Vcap) at 5500 V. The fragmentor was set at 85 V for all ions.

Calibration curves for ITZ, OH-ITZ and keto-ITZ were prepared between concentrations 1 nM and 1000 nM. For ND-ITZ the calibration curve was between 1 nM and 500 nM. The limit of detection for ITZ and its metabolites was 1 nM and the limit of quantification 5 nM. At a 50 nM nominal concentration, the accuracy for ITZ measurement was 99.2%, and the precision
(n=6) measured as %CV was 7.6%. For OH-ITZ measurements, the accuracy at a 50 nM nominal concentration was 100.5%, and the precision (n=6) was 5.0%. The accuracy of keto-ITZ measurement at 50 nM was 102.4% and the precision was 7.4% (n=6).

Nonspecific Binding of ITZ, OH-ITZ, Keto-ITZ and ND-ITZ in Human Liver Microsomes and Supersomes

The nonspecific protein binding of ITZ, OH-ITZ, keto-ITZ and ND-ITZ was studied using the tube absorption method described by Ishigam et al., 2001 (Ishigam et al., 2001). The method utilizes the propensity of basic lipophilic drugs to adsorb to experimental materials such as the inner wall of an incubation tube. The concentrations of the test compounds were measured by the same LC-MS method as described for microsomal incubations. Two sets of solutions, one with microsomal protein and one without microsomal protein, were prepared. To these, solutions of ITZ, OH-ITZ, keto-ITZ or ND-ITZ in acetone were added to achieve a final volume of 300 µl (a samples) or 200 µl (b samples) of 100 mM KPi buffer (1 mM EDTA, pH 7.4). The final acetone concentration was fixed at 1%. For Supersomes, ITZ and OH-ITZ were added at nominal concentrations of 25, 50, 250, 500 and 1000 nM. For liver microsomes, all compounds were added to produce a nominal concentration of 50, 250 and 500 nM. The protein concentrations were either 10 pmol CYP3A4/ml (0.085 mg protein/ml) of Supersomes or 0.025 mg/ml for pooled (five livers selected for the study) HLM. The solutions were vortexed and incubated at 37°C for 60 min. After the 60 min incubation, either a 200 µl aliquot was transferred to another tube containing 200 µl acetonitrile (sample a) or 200 µl acetonitrile was added into a tube containing the test compound in 200 µl of KPi buffer (sample b). All samples were mixed and centrifuged (12,000 g, 10 min) and the supernatant fraction was transferred to an autosampler vial and analyzed by LC-MS. From (a) samples, the concentration not adsorbed to the tube wall (C_u for samples containing only buffer and drug and C_m for samples containing
microsomal protein) was measured. From (b) samples, the total drug concentrations in the tube were measured (C_t for buffer samples and C_{t(m)} for microsomal or supersomal samples). The drug concentration adsorbed to the tube wall (C_w) was calculated from the buffer containing samples as follows:

\[ C_w = C_t - C_u \]  

(1)

The ratio of the unbound concentration to that adsorbed to the wall (r, partitioning constant for the wall) was calculated from the quotient of C_u and C_w. The concentration adsorbed to the tube wall when the drug was mixed with microsomes or Supersomes was calculated as:

\[ C_{w(m)} = C_{t(m)} - C_m \]  

(2)

The unbound concentrations in microsomal suspension (C_{u(m)}) were calculated from:

\[ C_{u(m)} = r_{\text{mean}} \cdot C_{w(m)} \]  

(3)

In which r_{\text{mean}} represents the mean value of r obtained at varying substrate concentrations. The r value was found to be independent of substrate concentration over the range of interest. The unbound fraction for microsomes (f_{u(m)}) was calculated using the equation:

\[ f_{u(m)} = \frac{C_{u(m)}}{C_m} \]  

(4)

All measurements were conducted in triplicate.

**Inhibition of CYP3A4 in Human Liver Microsomes and Supersomes by ITZ and Its Metabolites**

*Microsomal experiments:* The IC_{50} value for ITZ, OH-ITZ, keto-ITZ and ND-ITZ in human liver microsomes, was measured using 1’-hydroxylation of midazolam (OH-MDZ) as a probe reaction for CYP3A4-mediated metabolism. All the human liver microsomes used were known not to express appreciable levels of CYP3A5. The incubations were performed in 100 mM KPi buffer
(pH 7.4) with 1 mM EDTA. The NADPH concentration in all incubations was 1 mM. Pooled human liver microsomes were used with a final protein concentration of 0.025 mg/ml. The midazolam concentration was 1 µM in all incubations. ITZ, OH-ITZ and keto-ITZ were added to the 500 µl incubations at nominal concentrations of 0, 5, 25, 50, 100, 250, 500 and 1000 nM. After preincubation for 4 min at 37°C, a time 0 min sample (200 µl) was collected into a vial containing 200 µl of acetonitrile and the reaction was initiated with NADPH (final concentration 1 mM) and allowed to proceed for 2 min. At 2 min another 200 µl sample was taken and the reaction quenched with 200 µl of ice-cold acetonitrile. Ten µl of internal standard solution (D2-labeled OH-MDZ, 100 ng/ml) were added. All incubations were carried out in duplicate. The inhibitor concentration was measured at time 0 min and at time 2 min and the mean inhibitor concentration was calculated for the incubation period (i.e. the inhibitor concentration was corrected for depletion). OH-MDZ was measured only in the 2 min sample.

OH-MDZ concentrations were measured using a Hewlett Packard series 1100 MSD system operating at the positive ion electrospray mode with selected ion monitoring. The mobile phase consisted of methanol and water, both with 0.1% acetic acid with a gradient elution. Twenty µl of the sample were injected into the HPLC. The mobile phase flow was 0.25 ml/min and the initial mobile phase was 45% methanol and 65% water. The methanol concentration was increased linearly to 60% over 2 min. After 0.5 min, the methanol concentration was increased to 90% over 1.5 min and kept there for 1 min. At 5 min, the methanol concentration was decreased back to 45%. Separation was achieved using a Zorbax Eclipse XDB-C8 5µm column (2.1 mm i.d. × 50 mm; Agilent Technologies) equipped with a Phenomenex SecurityGuard C8 guard column. Ion m/z 342 was monitored for OH-MDZ and m/z 346 for the D2-labeled OH-MDZ (Cl37-isotope). For the mass spectrometer, the drying gas flow was 10 l/min, nebulizer pressure 25 psig, gas temperature 300°C and the capillary voltage (Vcap) 5000 V. The fragmentor was set at 110 V for all ions. The OH-MDZ calibration curve was prepared over a concentration range (10
different concentrations) between 1.2 and 350 ng/ml. The limit of quantification for OH-MDZ was 1.2 ng/ml. At mid-concentration (50 ng/ml) the accuracy of the OH-MDZ measurement was 101.8 %, and the precision of the assay was 8.9%.

**Supersome experiments:** The inhibition constant (K_i) for ITZ and OH-ITZ was determined using heterologously expressed CYP3A4 (Supersomes). The incubation mixture consisted of 5 pmol (10 pmol/mL) of cDNA-expressed CYP3A4 (0.084 mg/mL of total microsomal protein), midazolam (at concentrations 1, 2, 4 and 8 μM) and either ITZ or OH-ITZ (at nominal concentrations 0, 50, 100, 500 and 1000 nM) in 500 μL of 100 mM KPi buffer (pH 7.4) with 1 mM EDTA. After preincubation for 4 min at 37°C, a time 0 min sample (200 μL) was collected into a vial containing 200 μl of acetonitrile and the reaction was initiated with NADPH (1 mM final concentration) and allowed to proceed for 2 min. At 2 min, another 200 μl sample was taken and quenched into 200 μl of ice-cold acetonitrile and 10 μl of internal standard solution (D2-OH-MDZ, 100 ng/ml) were added. All incubations were carried out in duplicate. The acetonitrile-quenched samples were analyzed using the LC-MS methods described above for ITZ, OH-ITZ and keto-ITZ in both the 0 min and 2 min samples, whereas OH-MDZ was quantified only from the 2 min samples. The inhibitor concentrations in all incubations were again corrected for inhibitor depletion.

**Calculation of Enzyme Kinetic Parameters**

The Michaelis constant (K_m) was determined by the method described by Obach and Reed-Hagen (Obach and Reed-Hagen, 2002). A first-order decay function was fit to the data from logarithm of the percentage of the substrate remaining versus time at each substrate concentration to determine initial substrate depletion rate constants (k_{dep}). This parameter is equivalent to the apparent clearance (v/[S]) of the drug at any given concentration. Only the
initial time points, wherein log-linearity was observed, were used to determine depletion rate constants. $K_m$ values were determined from the expanded Michaelis-Menten equation by fitting the $k_{dep}$ versus the substrate concentration on a linear-log plot to the equation

$$k_{dep} = k_{dep[S \rightarrow 0]} \cdot (1 - \frac{[S]}{[S] + K_m}) \quad (5)$$

in which $[S]$ is the substrate concentration, $k_{dep[S \rightarrow 0]}$ represents the theoretical maximum substrate consumption rate under the experimental conditions and $K_m$ is the Michaelis constant (Sigmaplot, SPSS Inc., Chicago, IL).

The intrinsic clearance ($Cl_{int}$) of each substrate was determined from the maximum depletion rate constant ($k_{dep[S \rightarrow 0]}$) by dividing this value by the enzyme concentration in the incubation:

$$Cl_{int,u} = \frac{k_{dep[S \rightarrow 0]}}{C_{CYP3A4}} \quad (6)$$

The maximal substrate consumption velocity, $V_{max}$, was obtained using the relationship between $Cl_{int}$, $Cl_{int,u}$, $K_m$ and $V_{max}$:

$$V_{max} = Cl_{int,u} \cdot K_m \quad (7)$$

For this approach the unbound $K_m$ was used and the $Cl_{int,u}$ was calculated as described by Equation 6.

The inhibition parameters were determined by use of nonlinear regression analysis. Standard competitive and noncompetitive inhibition model was fit to the experimental data. (Systat 5.04, Systat Inc., Chicago, IL).

**RESULTS**

**Identification of ITZ Metabolites Formed by CYP3A4**
The first goal of this study was to confirm that ITZ is a substrate for CYP3A \textit{in vitro} and to characterize the metabolites generated. In preliminary studies with heterologously expressed CYP3A4 it was found that CYP3A4 metabolized ITZ efficiently to the primary metabolite OH-ITZ. An unknown metabolite with \textit{m/z} 719 [MH$^+$] was detected from the same ITZ-CYP3A4 incubations. MH$^+$ ions corresponding to a chlorine isotope pattern with two chlorine atoms were observed at \textit{m/z} 719 and \textit{m/z} 721. Based on the molecular weight, MS-MS fragmentation pattern (Figure 3) and comparison of retention times (Figure 2) and MS spectrum of the produced metabolite to a synthetic reference material, this unknown metabolite produced by CYP3A4 was identified as the corresponding ketone of OH-ITZ (keto-ITZ, Figure 1). Incubation of this new metabolite showed that it too was a substrate of CYP3A4 and efforts were made to identify additional downstream metabolites. A product with a shorter retention time than OH-ITZ (Figure 2) and MH$^+$ ions at \textit{m/z} 649 and \textit{m/z} 651 corresponding to the Cl isotope pattern of the molecule was detected. The confirmation for the MH$^+$ ions at \textit{m/z} 649 and \textit{m/z} 651 was obtained from detection of sodium and potassium adducts (\textit{m/z} 671, 673 and \textit{m/z} 687 and 689 respectively) and a M$^{2+}$ ion at \textit{m/z} 325. Based on a comparison to the synthetic reference material this metabolite was identified as N-desalkyl-itraconazole (ND-ITZ, Figure 1). This metabolite was also detected in incubations of ITZ and OH-ITZ with CYP3A4 (Figure 2). MS/MS characterization of the metabolically produced compound further confirmed the structure (Figure 3).

Qualitatively, based on the metabolite identification data, a sequential metabolic pathway for ITZ with CYP3A4 was established (Figure 4). In this metabolic pathway ITZ first undergoes hydroxylation to OH-ITZ and then a second oxidation to keto-ITZ. Keto-ITZ is further oxidized to undergo N-dealkylation to ND-ITZ. Because ND-ITZ was measured also in incubations with ITZ and OH-ITZ at saturating substrate concentrations, the possibility that N-dealkylation occurs directly from ITZ and/or from OH-ITZ cannot be excluded.
Only CYP3A4 and CYP1A1 catalyzed the metabolism of ITZ to OH-ITZ. In addition, none of the CYP isoforms, except CYP3A4, generated keto-ITZ or ND-ITZ. With respect to CYP1A1 and OH-ITZ formation, the rate of metabolism (500 nM, nominal conc) was approximately 3 pmol·min⁻¹·nmol⁻¹ 1A1 and no significant substrate depletion was observed. The combined formation velocity for OH-ITZ and keto-ITZ catalyzed by CYP3A4 was approximately 15 pmol·min⁻¹·nmol⁻¹ 3A4, but in this experiment significant depletion of ITZ was observed (>50%), which may have masked the true reaction velocity.

It was of interest to note that CYP3A5 (with or without cytochrome b5 supplementation) did not catalyze OH-ITZ formation, nor was there any evidence of significant metabolism to alternative products, as indicated by a flat substrate disappearance curve over 30 min of incubation. This was in marked contrast to a 44% and 20% depletion of ITZ at concentrations 50 nM and 500 nM respectively when it was incubated with CYP3A4 under the same conditions.

**Kinetic Analysis of ITZ Metabolism**

The enzyme kinetic parameters for ITZ metabolism were estimated using a substrate depletion approach. This method was adopted because ITZ appears to undergo sequential metabolism to multiple metabolites and significant substrate depletion was observed even at incubation times of 1-2 min. Figures 5a and 5b show the concentration versus time profiles for ITZ, OH-ITZ and keto-ITZ following incubation of 30 and 600 nM ITZ with CYP3A4. An early rapid depletion of ITZ was accompanied by the appearance of OH-ITZ and keto-ITZ. ND-ITZ concentrations in these incubations were below limits of quantification. At the lower ITZ concentrations, the concentration of OH-ITZ and keto-ITZ appeared to reach a maximum and subsequently decline with time, indicating that the two metabolites were also substrates of CYP3A4. Moreover, at the lowest ITZ concentration, there was a failure to achieve mass-
balance with an accounting of the detectable metabolites, suggesting that further biotransformation occurred.

In order to evaluate further the sequential metabolism of ITZ, substrate depletion experiments with OH-ITZ (Figure 5c and 5d) and keto-ITZ (Figure 5e and 5f) were performed. Keto-ITZ and ND-ITZ appeared to be primary metabolites of OH-ITZ and keto-ITZ, respectively. At the lowest substrate (OH-ITZ) concentrations tested, the concentration of keto-ITZ rose to a maximum and declined thereafter over time and mass-balance was again not achieved. The concentrations of ND-ITZ in incubations of ITZ and OH-ITZ were not measurable (below limit of quantification). In incubations with keto-ITZ, ND-ITZ could be quantified but mass balance was not obtained (Figure 5e and 5f). In incubations of ITZ and OH-ITZ with high enzyme (CYP3A4) concentrations (100 pmol/ml) ND-ITZ was also quantifiable. Preliminary experiments incubating ND-ITZ with CYP3A4 showed that it is also further depleted by CYP3A4 (data not shown).

For all three substrates (ITZ, OH-ITZ and keto-ITZ), biphasic depletion curves were observed (not shown). This biphasic behavior was most pronounced at the lowest substrate concentrations. For kinetic parameter estimation, only the initial log-linear portion of the depletion curve was used. A simple hyperbolic equation (eq. 5) was fit to the substrate depletion data to obtain the Michaelis constants (K_m) and the theoretical maximum depletion rate constants (k_{dep}^{max}) for ITZ, OH-ITZ and keto-ITZ with CYP3A4. A summary of the resulting parameters is presented in Table 1. The relationship between the first-order rate constant for substrate depletion (k_{dep}) and the substrate concentration [S] for each of the compounds is shown in Figure 6. All three molecules had similar unbound K_m values at the low nanomolar range, showing that ITZ, OH-ITZ and keto-ITZ all have a high affinity to CYP3A4. The turnover rates of ITZ, OH-ITZ and keto-ITZ were relatively slow, <1 min^{-1}, consistent with a “high affinity-low capacity”
classification. Interestingly, OH-ITZ had the lowest unbound intrinsic clearance of the studied compounds, and it is known to accumulate to levels above that of ITZ in vivo.

**Inhibition of CYP3A4 by ITZ, OH-ITZ and Keto-ITZ**

The inhibition constants for ITZ and OH-ITZ were measured using CYP3A4 Supersomes and midazolam as a probe substrate. Figure 7 shows the Lineweaver-Burk and Dixon plots for inhibition of OH-MDZ formation by ITZ and OH-ITZ. Inspection of the reciprocal plots suggests that ITZ and OH-ITZ are competitive inhibitors of CYP3A4. This was confirmed by nonlinear regression analysis using alternative models. For ITZ, a small but insignificant noncompetitive component was detected as well using nonlinear regression. The kinetic parameters for CYP3A4 inhibition are summarized in Table 2. Both ITZ and OH-ITZ appeared to be high affinity inhibitors of CYP3A4, based on the low nanomolar unbound \( K_i \).

In incubations of CYP3A4 Supersomes with MDZ and ITZ, up to 40% of the inhibitor pool was depleted during the 2-min incubation period and OH-ITZ could be quantified in all incubation products. For reaction mixtures with relatively high initial ITZ concentrations, keto-ITZ could be detected as well. Depletion of OH-ITZ during MDZ incubation was significantly less than that of ITZ, 5-10%, and observed only at the low initial OH-ITZ concentrations. The measured concentrations of OH-ITZ and keto-ITZ as metabolites were used to evaluate the possible contribution of these metabolites to the inhibition of midazolam hydroxylation. OH-ITZ was present at levels 10 times lower than the ITZ concentration when ITZ was used as an inhibitor. At the highest ITZ concentration employed, OH-ITZ concentrations rose to concentrations equal to its \( K_i \) at 2 min, whereas in the incubation with ITZ at 280 nM, OH-ITZ concentrations at 2 min were 0.5 times its \( K_i \). At the lowest ITZ concentration, OH-ITZ concentrations were approximately 10-fold below its measured \( K_i \). When OH-ITZ was used as the inhibitor, keto-ITZ concentrations were 4-10 times lower than those of OH-ITZ. Based on
these results it is unlikely that OH-ITZ and keto-ITZ contributed by a reversible mechanism to CYP3A4 inhibition following incubation with ITZ under the in vitro conditions employed. However, because of the partial depletion of ITZ at the lowest inhibitor concentrations, we cannot rule out possible contribution from unknown ITZ metabolites to the overall inhibitory effect. It is also possible that there could be slowly reversible or irreversible CYP3A4 inhibition following incubation with ITZ, OH-ITZ, keto-ITZ or ND-ITZ from the production of relatively small amounts of an unknown downstream ITZ metabolite(s).

The inhibitory potency of ITZ, OH-ITZ, keto-ITZ and ND-ITZ was confirmed in experiments with pooled human liver microsomes. Figure 8 shows a plot of the inhibitor concentration versus percent inhibition. All three compounds gave a maximum inhibition of >90% at the highest nominal concentration tested (1 µM for all compounds except ND-ITZ which was 650 nM). At the lowest nominal inhibitor concentration, a small but consistent activation of CYP3A4 was observed. The IC₅₀ values that were obtained are shown in Table 2. Interestingly ND-ITZ was a more potent inhibitor of CYP3A4 in human liver microsomes than any of the other tested compounds, which had equivalent inhibitory potencies. ITZ and OH-ITZ depletion was less than that seen in experiments with CYP3A4 Supersomes. Only 5% of ITZ was depleted at the highest concentration used and 25% of ITZ was depleted at the lowest concentration. In addition, there was no measurable depletion of OH-ITZ, keto-ITZ or ND-ITZ under the liver microsomal incubation conditions involved. This difference between the Supersome and human liver microsome experiments is attributed to the lower amount of active CYP3A4 available in microsomal incubations.

**Effect of Nonspecific Microsomal Binding on Inhibition Parameters**

Nonspecific protein binding of ITZ, OH-ITZ, keto-ITZ and ND-ITZ was measured in Supersomes and liver microsomes. All of the test compounds were bound extensively, even at
the low protein concentrations of 0.025-0.087 mg/ml that were employed. The unbound fraction of the test compounds were between 10-20% with the exception of OH-ITZ in Supersomes (38%) and ND-ITZ in microsomes (2.6%) (Tables 1 and 2), and the bound fraction appeared to be independent of the nominal inhibitor concentration (data not shown). Accordingly, the extensive protein binding had a significant effect on the observed $K_m$ and $K_i$ values and must be taken into account when in vitro- to in vivo predictions are made. Also, the high lipophilicity of ITZ and its metabolites, their poor solubility in aqueous buffers, high protein binding and low enzymatically relevant nominal concentrations make it extremely difficult to estimate the free fraction for ITZ and its metabolites accurately.

**DISCUSSION**

Clinical evidence suggests that itraconazole (ITZ) is a substrate of CYP3A4. The results of this study confirmed that hydroxy-itraconazole (OH-ITZ) is generated from ITZ by CYP3A4. In addition, it was discovered that CYP3A4 further catalyzes the conversion of OH-ITZ to the corresponding ketone, keto-itraconazole (keto-ITZ), and also catalyzes the dealkylation of the keto-ITZ and possibly ITZ and OH-ITZ, to generate N-desalkyl-itraconazole (ND-ITZ). These sequential oxidations may occur via two different mechanisms as suggested previously for testosterone and levo-α-acetyl-methadol (Sugiyama et al., 1994; Oda and Kharasch, 2001). Either the primary metabolite-enzyme complex, OH-ITZ-CYP3A4, is activated and converted to the secondary metabolite, keto-ITZ before OH-ITZ is released from the enzyme or OH-ITZ is released from the enzyme and then re-associates with the enzyme to be oxidized to keto-ITZ. In the first scenario, the ratio of the keto-ITZ formation rate to the OH-ITZ formation rate will be constant with varying ITZ concentrations, whereas for the second scenario, the formation of keto-ITZ will eventually cease when enzyme becomes saturated with the initial substrate, ITZ, (i.e., OH-ITZ will not re-associate after dissociation). Results from this study suggest something
intermediate; keto-ITZ was detected both in incubations with ITZ at saturating concentrations (1 µM) and in incubations of high concentrations of ITZ and midazolam. This suggests that at least part of the keto-ITZ product must be generated from ITZ without dissociation of OH-ITZ from CYP3A4.

Two new metabolites of ITZ formed by CYP3A4 were identified in this study. Mass spectrometric and chromatographic data and comparison to authentic standards supports the identification of these metabolites as keto-ITZ and ND-ITZ. ND-ITZ has been previously reported as a urinary and fecally excreted metabolite of itraconazole in rats and dogs (Heykants et al., 1987). Since ITZ is not excreted unchanged into the urine and only a small fraction of the dose (5%) is excreted unchanged in the feces, the three metabolites described here may contribute significantly to the urinary recovery of ITZ. At present it is not known if keto-ITZ and ND-ITZ circulate in plasma in vivo, but due to the sequential, partly nondissociative nature of ITZ metabolism by CYP3A4 to keto-ITZ via OH-ITZ, one could expect that significant amounts of keto-ITZ would be formed in vivo. However, the ketone metabolite may be rapidly reduced to the alcohol by reductase enzymes, resulting in a disproportionate accumulation of OH-ITZ as CYP3A4 activity is inhibited with multiple ITZ dosing. There is also the possibility of N-dealkylation of keto-ITZ by CYP3A4.

The biphasic elimination of ITZ observed at all nominal in vitro concentrations mimics the elimination profile observed in vivo. The biphasic elimination of ITZ in vivo has thus far been explained by distribution kinetics. However, it is unlikely that distribution accounts for the biphasic elimination in the expressed enzyme system. Recently, it was reported that the stereoisomers of ITZ have significantly different steady-state plasma concentrations (Breadmore and Thormann, 2003). Thus, stereoselective clearance of the four ITZ stereoisomers (two enantiomeric pairs) by CYP3A4 would be a plausible explanation for the biphasic elimination of total ITZ observed in our in vitro incubations and in vivo. A set of high clearance stereoisomers
(pair of enantiomers) might account for the initial fast elimination phase and a set of low clearance enantiomers attributing to the second slower elimination phase. Alternatively, the biphasic depletion of ITZ \textit{in vitro} and \textit{in vivo} could involve gradual loss of enzyme activity by time-dependant inactivation of the enzyme or inhibition of the enzyme by accumulating metabolites.

Results from a previous study by Yamano et al. (Yamano et al., 2001) suggest that ITZ does not cause irreversible time-dependant inactivation of CYP3A4 when incubated with human liver microsomes. However, this finding may be incubation condition-dependent, particularly in view of the apparent high affinity of downstream ITZ metabolites such as ND-ITZ for CYP3A4. It is possible that metabolism of ND-ITZ may eventually result in a metabolite-enzyme complex that is so stable (towards further metabolism and dissociation) as to effectively preclude any productive metabolism of other CYP3A4 substrates. Preliminary experiments provided us with no convincing evidence that classical mechanism-based inhibition occurs, but more careful experimentation is needed to evaluate this mechanistic possibility.

ITZ exhibits disproportional increase in AUC with increasing single oral doses and a time-dependent reduction in oral clearance with multiple dosing (Hardin et al., 1988; Heykants et al., 1989; Haria et al., 1996). In addition, ITZ bioavailability is dose-dependant (Schäfer-Körting, 1993) and ITZ elimination was found to follow Michaelis-Menten kinetics \textit{in vivo} (Barone et al., 1993). Given our \textit{in vitro} findings, concentration-, dose- and time-dependent kinetic nonlinearity may be a consequence of saturation of intestinal and hepatic CYP3A4 by ITZ metabolites, in addition to the parent drug.

A number of investigators have evaluated the inhibitory potency of ITZ towards CYP3A4 in human liver microsomes. The probe substrates have included cyclosporine (Back and Tjia, 1991), midazolam (von Moltke et al., 1996; Wang et al., 1999), diazepam (Tran et al., 2002), and simvastatin (Ishigam et al., 2001). Using midazolam as a probe, two different investigators...
obtained a 10-fold difference in the $K_i$ values for ITZ, 2300 nM (Wang et al., 1999) and 270 nM (von Moltke et al., 1996). However, the microsomal protein content used in the studies was 0.25 mg/ml. Using cyclosporine as a probe and 0.15 mg/ml microsomal protein, an ITZ $K_i$ of 700 nM was reported (Back and Tjia, 1991). More recently, Ishigam et al. (Ishigam et al., 2001) obtained a range of $K_i$ values of 27-93 nM for ITZ using simvastatin as a probe and 0.04 mg/ml microsomal protein. These studies show a trend towards a lower $K_i$ measurement with decreasing protein concentrations, reflective of significant nonspecific microsomal protein binding and possibly inhibitor depletion. Indeed, the study by Tran et al. (Tran et al., 2002) showed a consistent decrease in the inhibitory potency of ITZ with increasing protein concentrations supporting the theory that nonspecific microsomal binding affects ITZ’s $in vitro$ $K_i$. The study by Ishigam et al. (Ishigam et al., 2001) also measured the unbound liver microsomal $K_i$ for ITZ, which was 1.4-4.7 nM. The results presented here for ITZ ($K_i$ 15.7 nM and $K_{iu}$ 1.3 nM) using expressed CYP3A4, are similar to their data, although slightly lower in magnitude.

Previous investigators have also evaluated the inhibitory potency of OH-ITZ towards CYP3A4 in human liver microsomes using MDZ, triazolam or diazepam as a substrate probe (von Moltke et al., 1998; Wang et al., 1999; Tran et al., 2002). For two of the studies, (von Moltke et al., 1998; Wang et al., 1999), OH-ITZ was a slightly less potent inhibitor of CYP3A4 than ITZ (1.2- to 2.7 fold), and for the other (Tran et al., 2002) it was 2-fold more potent than ITZ. The reported $K_i$ or $IC_{50}$ values for OH-ITZ varied between 95 - 6300 nM. These parameter values illustrate the substantial affinity of OH-ITZ for CYP3A4, but do not adequately explain the observed $in vivo$ inhibitory effect of ITZ after consideration of expected unbound circulating OH-ITZ plasma concentrations. In contrast, the unbound $K_i$ and $IC_{50}$ of OH-ITZ determined in this study are at least 7-fold lower than previously reported parameter values and make a much stronger case for a significant contribution from OH-ITZ to the $in vivo$ inhibitory effect.
In addition to OH-ITZ, which is known to circulate at higher concentrations than ITZ in plasma (Heykants et al., 1989; Barone et al., 1993; Poirier and Cheymol, 1998), the new metabolites identified in this study, keto-ITZ and ND-ITZ, may contribute to the increased inhibition of CYP3A4 by ITZ with longer therapy. Some investigators have observed an unusually persistent inhibition of CYP3A4-mediated metabolism of tacrolimus and cyclosporine by ITZ after cessation of ITZ administration (Trenk et al., 1987; Cervelli and Russ, 2003). This prolonged inhibitory effect, that exceeds in duration four half-lives of ITZ, could be explained by circulating metabolites of ITZ that have a half-lives that exceed that of the parent drug, or slow dissociation of an inhibitory metabolite-enzyme complex. The fact that ND-ITZ was the most potent inhibitor of the tested compounds in this study and it has been detected as a urinary metabolite of ITZ in humans and animals, would support the hypothesis that this metabolite contributes significantly to the persistent inhibition of CYP3A4 in vivo.

ITZ is a triazole antifungal agent that is traditionally believed to bind to the fungal P450 by coordinating the heme via the triazole ring nitrogen. This has also been the supposed mechanism for CYP3A4 inhibition. However, the close agreement of the determined $K_{i,u}$ and $K_{m,u}$ values suggests an alternative hypothesis. Coordination to the heme-iron of CYP3A4 by ITZ is not a prerequisite for inhibition, but instead inhibition of CYP3A4 may occur due to its metabolism via this enzyme at the opposite end of the substrate/inhibitor molecule. (This assumes that metabolism of ITZ to OH-ITZ cannot occur if the triazole nitrogen is coordinated to the heme iron). Further experiments are underway to explore this possibility.

Results from this investigation do not exclude the possibility that there is active, concentrative uptake of ITZ into human hepatocytes and that this process contributes to the apparent potency of ITZ as an inhibitor of CYP3A4 in vivo. Data to support this mechanistic explanation come from rat liver experiments and confirmation with human hepatocytes is needed.
Ultimately, the profound effects of ITZ therapy on CYP3A-catalyzed drug clearance may be multi-factorial.

In conclusion, this study has shown that ITZ and its subsequent sequential metabolites (OH-ITZ, keto-ITZ and ND-ITZ) are all high affinity ligands of CYP3A4 and they undergo metabolism by CYP3A4. These metabolites may contribute to CYP3A4 inhibition in vivo following ITZ therapy. The results of this study also provide insight into the reasons for underprediction of CYP3A4 inhibition by ITZ in vivo from in vitro Kᵢ data and suggest that nonspecific protein binding, accumulation of inhibitory metabolites and inhibitor depletion can contribute to mispredictions.

ACKNOWLEDGMENTS

This study was supported in part by NIH PO1 GM32165.
REFERENCES


LEGENDS FOR FIGURES

Figure 1. Chemical structures of ITZ, OH-ITZ, keto-ITZ and ND-ITZ.

Figure 2. Selected mass spectrometric ion chromatograms from incubations of (a) ITZ, (b) OH-ITZ (c) and keto-itraconazole with heterologously expressed CYP3A4. Identified peaks were ITZ, OH-ITZ, keto-ITZ and ND-ITZ. In (a) and (b) ND-ITZ ion chromatogram is expanded 10-fold. Substrate concentrations in incubations were 1000 nM and incubation conditions were as described in experimental for identification of metabolites.

Figure 3. Tandem mass spectrometric (MS/MS) characterization of ITZ metabolites. MS/MS spectras of (a) ITZ standard using ion m/z 705; (b) OH-ITZ standard using ion m/z 721; (c) metabolically produced keto-ITZ using ion m/z 719; (d) metabolically produced ND-ITZ using ion m/z 649 and (e) suggested fragmentation pattern for ITZ, OH-ITZ, keto-ITZ and ND-ITZ. Incubation conditions for generating keto-ITZ and ND-ITZ were as described in experimental methods. ITZ and OH-ITZ spectra were obtained from a control incubation in which no NADPH was added.

Figure 4. Suggested sequential CYP3A4-catalyzed metabolic pathway for ITZ. Rate constants for metabolism (k_{cat}) and release of ITZ and metabolite intermediates (K_D) are included to illustrate the possibility for non-dissociative or dissociative sequential metabolism.

Figure 5. Concentrations of ITZ, OH-ITZ and keto-ITZ in incubations of ITZ (a and b), OH-ITZ (c and d) and keto-ITZ (e and f) with heterologously expressed CYP3A4. ITZ concentrations were 28 nM (a) and 580nM (b), OH-ITZ concentrations were 25 nM (c) and 337 nM (d) and
keto-ITZ concentrations were 37 nM (e) and 923 nM (f). Incubation conditions were as described in experimental methods. ♦ - total calculated concentration, ● - measured ITZ, ■ - measured OH-ITZ, ▲ - measured keto-ITZ and ○ - measured ND-ITZ.

Figure 6. Concentration versus first-order depletion rate constant (k_{dep}) plots and determination of Michaelis constants (with data nonlinearly fitted to equation 5) for ITZ (a), OH-ITZ (b) and keto-ITZ (c), using the substrate depletion approach. Depletion rate constant for each substrate concentration was obtained as described in experimental methods using a linear fit of log percent remaining versus time plots.

Figure 7. Lineweaver-Burk plots for the inhibition of CYP3A4-mediated formation of 1-OH-midazolam by (a) ITZ and (b) OH-ITZ. Insets show the corresponding Dixon plots.

Figure 8. Nominal inhibitor concentration versus percent remaining activity plots for ITZ, OH-ITZ, keto-ITZ and ND-ITZ using pooled human liver microsomes and midazolam hydroxylation as a CYP3A4 probe reaction. ♦ - ND-ITZ, ● - ITZ, ■ - OH-ITZ and ▲ - keto-ITZ.
TABLES

Table 1. Kinetic parameters for cDNA-expressed CYP3A4 (Supersomes™) mediated metabolism of ITZ, OH-ITZ and keto-ITZ.

<table>
<thead>
<tr>
<th></th>
<th>$K_m \pm SD (nM)$</th>
<th>$f_u \pm SD (%)$</th>
<th>$K_{m,u} (nM)$</th>
<th>$k_{dep[S]} \rightarrow 0 \pm SD (1/min)$</th>
<th>$V_{max} (pmol/min/nmol 3A4)$</th>
<th>$Cl_{int,u} (ml/min/nmol CYP3A4)$</th>
</tr>
</thead>
<tbody>
<tr>
<td>ITZ</td>
<td>44.4 ± 11.1</td>
<td>8.8 ± 3.2</td>
<td>3.9</td>
<td>0.69 ± 0.07</td>
<td>270</td>
<td>69.3</td>
</tr>
<tr>
<td>OH-ITZ</td>
<td>71.7 ± 37.5</td>
<td>38 ± 20</td>
<td>27</td>
<td>0.33 ± 0.06</td>
<td>543</td>
<td>19.8</td>
</tr>
<tr>
<td>keto-ITZ</td>
<td>17.9 ± 8.8</td>
<td>9.2 ± 4.9</td>
<td>1.4</td>
<td>0.63 ± 0.18</td>
<td>86.9</td>
<td>62.5</td>
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</table>

SD, standard deviation
Table 2. Kinetic parameters for CYP3A4 inhibition by ITZ and its metabolites OH-ITZ, keto-ITZ, and ND-ITZ. $K_i$ values were calculated from experiments with heterologously expressed CYP3A4 Supersomes and IC$_{50}$ values were determined from inhibition of midazolam hydroxylation in pooled human liver microsomes. The 95% confidence interval for the parameter estimates is presented in brackets.

<table>
<thead>
<tr>
<th></th>
<th>$K_i$ (nM)</th>
<th>$K_{i,u}$ (nM)</th>
<th>IC$_{50}$ ± SE (nM) [95% CI]</th>
<th>$I_{50}$ ± SD (%)</th>
<th>IC$_{50}$ u (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ITZ</td>
<td>15.7</td>
<td>1.3</td>
<td>29 ± 10 [0-58]</td>
<td>19.6 ± 9.2</td>
<td>6.1</td>
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<tr>
<td>OH-ITZ</td>
<td>37.8</td>
<td>14.4</td>
<td>37 ± 13 [5-70]</td>
<td>12.4 ± 6.8</td>
<td>4.6</td>
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<tr>
<td>keto-ITZ</td>
<td>NA</td>
<td>NA</td>
<td>53 ± 17 [10-97]</td>
<td>11.7 ± 4.2</td>
<td>7.0</td>
</tr>
<tr>
<td>ND-ITZ</td>
<td>NA</td>
<td>NA</td>
<td>17 ± 6.6 [0-34]</td>
<td>2.6 ± 1.2</td>
<td>0.44</td>
</tr>
</tbody>
</table>

SE, standard error; SD, standard deviation; 95% CI, 95% confidence interval; NA, not available.
Figure 1

Itraconazole  \( R = \text{CH}_3\text{CH}_2\text{CH}_3 \)

Hydroxy-itraconazole  \( R = \text{CH}_3\text{CHCH}_3\text{OH} \)

Keto-itraconazole  \( R = \text{CH}_3\text{C}_=\text{CH}_3 \)

N-Desalkyl-itraconazole  \( R = \text{H} \)
Figure 2

a. ND-ITZ, OH-ITZ, and keto-ITZ peaks with m/z 649, 719, and 721 at various times.

b. ND-ITZ and OH-ITZ with m/z 649 and 719, and keto-ITZ with m/z 721.

c. ND-ITZ and keto-ITZ with m/z 649 and 719.
Figure 4

\[
\text{ITZ + CYP3A4} \xrightarrow{K_{D,1}} \text{ITZ-CYP3A4} \xrightarrow{k_{cat,1}} \text{OH-ITZ-CYP3A4} \xrightarrow{k_{cat,2}} \text{Keto-ITZ-CYP3A4} \xrightarrow{k_{cat,3}} \text{ND-ITZ-CYP3A4}
\]

\[
\text{OH-ITZ + CYP3A4} \xrightarrow{K_{D,2}} \text{Keto-ITZ + CYP3A4} \xrightarrow{K_{D,3}} \text{ND-ITZ + CYP3A4} \xrightarrow{K_{D,4}}
\]

Itraconazole → Hydroxy-itraconazole → Keto-itraconazole → N-desalkyl-itraconazole

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Figure 5
Figure 6

(a) ITZ concentration (nM)

(b) OH-ITZ concentration (nM)

(c) Keto-ITZ concentration (nM)
This article has not been copyedited and formatted. The final version may differ from this version.
Figure 8

![Graph showing inhibitor concentration (nM) vs. % remaining activity.](image-url)